Each number of Acta Pharmacologica et Toxicologica consists of about 5 printed sheets, 6 numbers forming a volume. The numbers will be issued as soon as the articles submitted can be printed.

In order to avoid unnecessary delay and trouble authors are urgently requested only to send in manuscripts prepared according to the rules given on the second page of the cover.

The subscription should be forwarded to the publishers: *Munksgaard, 6, Nørregade, DK-1165 Copenhagen K*, Denmark, or to any international bookseller. The price of each volume is 120 Danish Kroner plus 16.00 Kroner for postage ($19.05) payable in advance.

Three copies of the manuscripts should be sent to Acta Pharmacologica et Toxicologica, 20, Juliane Mariesvej, DK-2100 Copenhagen Ø, Denmark, either directly or through one of the editors named on page 1 of the cover.

ACTA PHARMACOLOGICA
ET TOXICOLOGICA
VOL. 29 FASC. 2-3

INDEX

Issued:

F. J. BERNDTS

EACH number of ACTA PHARMACOLOGICA ET TOXICOLOGICA consists of about 5 printed sheets, 6 numbers forming a volume. The numbers will be issued as soon as the articles submitted can be printed.

In order to avoid unnecessary delay and trouble authors are urgently requested only to send in manuscripts prepared according *to the rules given on the second page of the cover.*

The subscription should be forwarded to the publishers: MUNKSGAARD, 6, Nørregade, DK-1165 Copenhagen K, Denmark, or to any international bookseller. The price of each volume is 120 Danish Kroner plus 16.00 Kroner for postage ($19.05) payable in advance.

Three copies of the manuscripts should be sent to Acta Pharmacologica et Toxicologica, 20, Juliane Mariesvej, DK-2100 Copenhagen Ø, Denmark, either directly or through one of the editors named on page 1 of the cover.

ACTA PHARMACOLOGICA
ET TOXICOLOGICA

VOL. 29 FASC. 4

INDEX

Issued May 1971.

F.J.BENN

Each number of Acta Pharmacologica et Toxicologica consists of about *5 printed sheets, 6 numbers forming a volume.* The numbers will be issued as soon as the articles submitted can be printed.

In order to avoid unnecessary delay and trouble authors are urgently requested only to send in manuscripts prepared according to the rules given on the second page of the cover.

The subscription should be forwarded to the publishers: Munksgaard, 6, Nørregade, DK-1165 Copenhagen K, Denmark, or to any international bookseller. The price of each volume is 120 Danish Kroner plus 16.00 Kroner for postage ($ 19.05) payable in advance.

Three copies of the manuscripts should be sent to Acta Pharmacologica et Toxicologica, 20, Juliane Mariesvej, DK-2100 Copenhagen Ø, Denmark, either directly or through one of the editors named on page 1 of the cover.

Attention

The Fifth International Congress on Pharmacology will be held in San Francisco, California, July 23–28, 1972. Its purpose is to promote pharmacological knowledge and the application of this knowledge to the health and well-being of man throughout the world.

For Further Information, Write to:

Secretariat
Fifth International Congress on Pharmacology
9650 Rockville Pike
Bethesda, Maryland 20014, U.S.A.

ACTA PHARMACOLOGICA
ET TOXICOLOGICA

VOL. 29 FASC. 5-6

INDEX

Issued July 1971.

EACH number of ACTA PHARMACOLOGICA ET TOXICOLOGICA consists of about 5 printed sheets, 6 numbers forming a volume. The numbers will be issued as soon as the articles submitted can be printed.

In order to avoid unnecessary delay and trouble authors are urgently requested only to send in manuscripts prepared according to the rules given on the second page of the cover.

The subscription should be forwarded to the publishers: MUNKSGAARD, 6, Nørregade, DK-1165 Copenhagen K, Denmark, or to any international bookseller. The price of each volume is 120 Danish Kroner plus 16.00 Kroner for postage ($ 19.05) payable in advance.

Three copies of the manuscripts should be sent to Acta Pharmacologica et Toxicologica, 20, Juliane Mariesvej, DK-2100 Copenhagen Ø, Denmark, either directly or through one of the editors named on page 1 of the cover.

ATTENTION

The Fifth International Congress on Pharmacology will be held in San Francisco, California, July 23–28, 1972. Its purpose is to promote pharmacological knowledge and the application of this knowledge to the health and well-being of man throughout the world.

For Further Information, Write to:

SECRETARIAT
FIFTH INTERNATIONAL CONGRESS ON PHARMACOLOGY
9650 Rockville Pike
Bethesda, Maryland 20014, U.S.A.

Acta pharmacol. et toxicol. 1971, 29, 1–8.

From the Institute of Pharmacology, University of Oslo, Norway

The Action of Thioridazine and Promazine on Biological Membranes: Relationship between ATPase Inhibition and Membrane Stabilization

By

Knud Landmark* and Ivar Oye

(Received May 25, 1970)

Abstract: The effects of promazine and thioridazine on hypotonic haemolysis of human erythrocytes are compared with their effect on Na^+-K^+-ATPase of washed human erythrocyte ghosts. Promazine (5×10^{-5} – 5×10^{-4} M) and thioridazine (10^{-5} – 10^{-4} M) stabilize erythrocytes against hypotonic haemolysis, but have lytic effects at higher concentrations. Both drugs inhibit Na^+-K^+-ATPase of erythrocyte membranes. This inhibition is slight at drug concentrations which have a membrane-stabilizing action, but is complete and irreversible at lytic concentrations of the drugs. Promazine and thioridazine also inhibit Na^+-K^+-ATPase in a membrane fraction prepared from rat hearts. The relative order of potency of the two drugs in this respect does not reflect their relative potency as general cardiac depressants. It is concluded that Na^+-K^+-ATPase is not a primary target for the action of promazine and thioridazine in the rat heart. It is suggested that inhibition of Na^+-K^+-ATPase by these agents is secondary to more general alterations of the physical properties of the cell membrane.

Key-words: Promazine – thioridazine – phenothiazines – ATPase – haemolysis – heart – membrane stabilization.

Psychotropic phenothiazine derivatives inhibit the Na^+-K^+-activated adenosine triphosphatase (ATP phosphohydrolase, E. C. 3.6.1.3, called Na^+-K^+-ATPase in the following text) of particulate fractions of tissue homogenates, and this inhibition has been considered essential for their pharmacological action (DAVIES & BRODY 1966; KRAUS & ŠIMÁNĚ 1967; ROBINSON *et al.* 1968). The Na^+-K^+ATPase is involved in the active transport of Na^+ and K^+ across cell membrane (see review by SKOU 1965), and a decrease in the

* Research Fellow, The Norwegian Council on Cardiovascular Diseases. Present Address: Institute of Pharmacology, University of Oslo.

cellular K^+ content would therefore be expected when the Na^+-K^+-ATPase activity is inhibited. In the rat heart, however, phenothiazines in low concentrations increase rather than decrease the cellular K^+ content. Furthermore they reduce the K^+ loss from hearts perfused at low K^+ concentrations both in the working as well as in the asystolic state (LANDMARK 1971; LANGSLET 1970). The effect of these drugs on the performance of the working heart resembles that of quinidine, local anaesthetics and other "membrane stabilizing agents" as defined by SHANES (1958).

The phenothiazines also act as membrane stabilizers in non-excitable cells and subcellular particles. They protect erythrocytes against hypotonic and mechanical haemolysis (SEEMAN & WEINSTEIN 1966) as well as haemolysis caused by isotonic urea, glycerol (FREEMAN & SPIRTES 1963), lysolecithin (GREIG & GIBBONS 1955) and storage (HALPERN *et al.* 1958).

The phenothiazines thus apparently alter the physical properties of cell membranes. As a working hypothesis it may be suggested that the inhibition of Na^+-K^+-ATPase by phenothiazines is unspecific and secondary to these general alterations of the cell membrane. In order to test this hypothesis we have obtained dose-response curves for Na^+-K^+-ATPase inhibition by thioridazine and promazine in erythrocyte- and cardiac membrane fractions, and have related these curves to the dose-response curves for their effect on hypotonic haemolysis of erythrocytes as the well as on the effect as membrane stabilizers in the perfused heart.

Methods

Assay of ATPase activity. ATPase activity was measured basically according to POST *et al.* (1960) in an incubation system consisting of 0.25 ml of the membrane or particle suspension, 1 ml 100 mM TRIS-histidine buffer (pH 7.1), 0.5 ml 10 mM TRIS-ATP (pH 7.1), 0.5 ml 10 mM-$MgCl_2$ and 0.25 ml of either water or salt solution (0.33 M-KCl and 0.80 M-NaCl). The drugs were dissolved in aliquots of the same salt solution (or in water) and diluted with this solution to the desired concentrations. The tubes were incubated at 40° for 60 min., the reaction stopped by adding 1.5 ml ice-cold 8 per cent perchloric acid, and the precipitate removed by filtration. Samles of the filtrate were transferred to tubes containing 5 ml cold isobutanol/benzene (1 : 1), 0.5 ml acetone and 0.5 ml acid molybdate (FISKE & SUBBAROW 1925), mixed for 15 sec on a Vortex mixer and centrifuged. Two ml of the isobutanol phase was then mixed with 2 ml ethanol/sulphuric acid (32 ml conc. sulphuric acid in 968 ml absolute ethanol) and the blue colour of phosphomolybdate developed by adding 50 μl $SnCl_2$ (10 g freshly dissolved in 20 ml 18 N-H_2SO_4). The absorption was recorded spectrophotometrically at 625 nm. The enzyme activity was calculated as μmole inorganic phosphate produced per mg dried "membrane material".

Preparation of erythrocyte membranes. Preparation of erythrocyte membranes was done essentially according to POST *et al.* (1960). Three parts of human venous blood were mixed with one part of anticoagulant citrate solution (45 mM sodium citrate, 20

mM citric acid, 72 mM glucose) and centrifuged at 1500 × g for 10 min. The blood cells were washed by resuspension in 6 volumes cold 0.145 M-NaCl and centrifuged as mentioned above. This washing was repeated several times. The cells were haemolyzed by quickly adding deionized water to the packed cells during vigorous mixing and centrifuged at 20000 × g for 20 min. The sediment was suspended in histidine-imidazole buffer (5×10^{-4} M, pH 7.1) and centrifuged at 10000 × g for 10 min. This step was repeated five times. The "ghosts" were removed from the final pellet by decantation and the remaining dark "button" was discarded. The ghosts were stored on ice overnight and on the next day mixed with 0.1 M glycyl-glycine buffered with TRIS to pH 8.1. Ten volumes of water were added, and the suspension centrifuged at 16000 × g for 10 min. The precipitate was suspended in TRIS-glycylglycine (5×10^{-4} M, pH 7.0) and centrifuged again as above. This step was repeated 4–5 times. The white precipitate thus obtained was used for the assay of ATPase. The dry weight of the final suspension was about 6 mg/ml.

Preparation of a membrane fraction from heart muscle. Hearts were minced and homogenized with a Potter-Elvehjem type of homogenizer in 9 volumes of 0.32 M sucrose containing 0.5 mM EDTA. The homogenate was diluted with an equal amount of the NaI solution as described by NAKAO *et al.* (1963) giving final concentrations of 2.0 M-NaI, 2.5 mM cysteine, 5 mM-$MgCl_2$, and 4 mM-ATP. The mixture was briefly rehomogenized, incubated for 10 min. in ice-water and centrifuged for 20 min. 20000 × g. The supernatant was diluted with ice-cold water to 0.6 M-NaI, briefly rehomogenized and centrifuged again at 20000 × g for 20 min. The supernatant was discarded and the precipitate washed twice by resuspension and re-homogenisation in water followed by centrifugation. Finally the precipitate was suspended in cold water to the volume of the first sucrose homogenate, and stored at − 30° until used. The dry weight of the final suspension was about 2 mg/ml.

Resistance of erythrocytes to osmotic haemolysis. The haemolytic activity of the two drugs was studied in suspensions of human erythrocytes (5 per cent by packed cell volume in 0.45–0.50 % NaCl). The cells were incubated in the dark for 1 hour at 37°, and the amount of haemoglobin in the supernatant was measured spectrophotometrically at 410 nm. One hundred per cent haemolysis (for reference) was achieved by freezing and thawing the cell suspension. The pH was 7.15 and was not influenced by the addition of the drugs at concentrations below 2×10^{-3} M.

Results

Effect of thioridazine and promazine on erythrocytes and erythrocyte membranes.

Both thioridazine and promazine protect erythrocytes against hypotonic haemolysis, and the dose-response curves have the biphasic shape characteristic of membrane-stabilizing agents. When the concentration exceeds a certain level, the drugs act as potent lysins (fig. 1). The upper curve of fig. 1 shows the effect of thioridazine and promazine on the Na^+-K^+-ATPase activity of erythrocyte membrane fractions, prepared as described [illegible] "Methods". A slight but dose-dependent inhibition occurs [illegible] membrane-stabilizing concentrations of the drugs. At ly [illegible]

however, the inhibition of Na^+-K^+-ATPase is complete. The results presented in fig. 1 show that the inhibitory action of thioridazine and promazine on Na^+-K^+-ATPase is associated with general changes in the physical properties of the cell membrane.

In another series of experiments, membrane fractions were pre-incubated with 2.5×10^{-4} and 5×10^{-4} M promazine in 0.1 M histidine-imidazole buffer (pH 7.1) *for* 10 *min.* at 40°. *The incubate was divided in two aliquots* and one of these centrifuged at $16000 \times g$ for 15 min. and the membrane *material resuspended in histidine-imidazole buffer. This washing procedure* was repeated. Spectrophotometric absorption curves obtained from the supernatants as well as from the final enzyme suspension showed that the drugs were effectively removed by this washing procedure. The ATPase activities, however, were the same in the washed and unwashed membrane preparations, indicating that the inhibition of ATPase at these drug concentrations was irreversible.

Effect of thioridazine and promazine on the heart and heart muscle membrane fractions.

The effects of thioridazine and promazine on the performance of the perfused rat heart have been described in detail in a previous paper (LANDMARK *et al.* 1969). Changes in ECG pattern similar to those observed in patients receiving large therapeutic doses of phenothiazines occurred a·

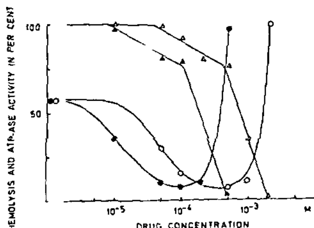


Fig. 1. Dose-response curves for the effects of promazine (open symbols) and thioridazine (filled symbols) on hypotonic haemolysis of human erythrocytes (circles) and Na^+-K^+-ATPase activity of washed erythrocyte ghosts (triangles). Each point is the mean of 4–6 experiments expressed in per cent of matched controls (14–18 μmole liberated inorganic phosphate per 100 mg dry weight/hr).

perfusate concentrations above 10^{-5} M of both drugs, and these ECG changes were associated with a decreased loss of K^+ from the heart (LANDMARK 1971; LANGSLET 1970).

In order to obtain Na^+-K^+-ATPase activity in fractions of cardiac muscle homogenates, it was necessary to prepare a "membrane fraction" as described under "methods". Crude particle fractions obtained by fractionation of sucrose or saline homogenates had an ATPase activity which was *inhibited* by Na^+ and K^+. In some preparations ouabain *stimulated* the ATPase activity of these crude fractions. The main ATP-hydrolyzing enzymes of crude cardiac muscle homogenates thus had some of the characteristics of the ATPase activity of actomyosin b. A Na^+-K^+-ATPase similar to that of erythrocyte membranes, however, could be obtained by treating the homogenate with concentrated salt solutions which presumably dissolve the contractile proteins. Fig. 2 shows dose-response curves for the inhibitory action of thioridazine and promazine on the Na^+-K^+-ATPase activities of cardiac membrane fractions, prepared as described under "methods". The Na^+-K^+-ATPase constitutes about 60 per cent of the total ATPase activity of these fractions. The inhibitory action of the two drugs was not specific for the Na^+-K^+-ATPase, although this ATPase was more sensitive to inhibition than the "basic" or "Mg^{++}-dependent" ATPase. Thioridazine was also a more potent inhibitor than promazine for cardiac ATPase. No inhibitory action was found at 10^{-5} M promazine, i. e. a concentration at which the drug has a marked cardio-depressive action on isolated rat atrial tissue (LANDMARK unpublished).

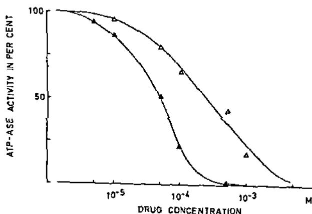


Fig 2. Dose-response curves for the effects of promazine (open triangles) and thioridazine (filled triangles) on Na^+-K^+-ATPase of a cardiac membrane fraction obtained rat hearts. Each point is the mean of 4–6 experiments expressed in per cent of controls. (61–242 μmole liberated inorganic phosphate per 100 mg dry wei

Discussion

An adenosine triphosphatase which is stimulated by Na^+ and K^+ was first described by SKOU (1957). Later investigations by POST *et al.* (1960), WHITTAM (1962), SKOU (1965) and others, provide evidence for a function of this enzyme in the active transport of Na^+ and K^+ across cell membranes. The Na^+-K^+-ATPase is inhibited by cardioactive digitalis glycosides which depress active ion transport in intact cells. The enzyme is also sensitive to a number of pharmacological agents classified as anaesthetics, hypnotics and tranquilizers (ISRAEL & SALAZAR 1967; UEDA & MIETANI 1967; TREVOR & CUMMINS 1969), and inhibition of brain Na^+-K^+-ATPase has been considered to be of importance for the pharmacological action of phenothiazines (DAVIS & BRODY 1966; KRAUS & ŠIMÁNĚ 1967; ROBINSON *et al.* 1968).

The pharmacological action of thioridazine and promazine in human erythrocytes and in the isolated rat heart, cannot be due to an inhibition of the Na^+-K^+-transport mechanism. Inhibition of Na^+-K^+-ATPase would be expected to cause a loss of K^+ from the cells. Both drugs, however, decreased K^+ loss from erythrocytes as well as from the heart (SEEMAN & WEINSTEIN 1966; LANDMARK 1971). The dose-response curves presented above, show that Na^+-K^+-ATPase inhibition is moderate at drug concentrations which stabilize cell membranes. On increasing the drug concentrations to levels which lyse cell membranes, the ATPase activity rapidly declines. In erythrocytes the dose-response curves for ATPase activity reflect the relative order of potency of the two drugs as haemolytic agents. The dose-response curve for cardiac ATPase inhibition, however, does not correlate with the relative order of potency of the two drugs as general cardiac depressants. In this respect promazine is more potent than thioridazine (LANDMARK unpublished). We therefore conclude that the inhibition of Na^+-K^+-ATPase does not play an essential role in the pharmacological action of thioridazine and promazine on the heart. The partial inhibition of Na^+-K^+-ATPase at drug concentrations which stabilize the cell membrane and reduce K^+ loss, might reflect a stabilization of membrane macromolecules against conformational changes necessarily involved in processes like excitation, impulse propagation and activation of membrane enzyme complexes. The molecular basis for this stabilization is unknown, but might involve widely different mechanisms such as alteration of the membrane lipid phase (CHOWDHURY *et al.* 1969), charge transfer reactions or clatrate formation (PAULING 1961).

The complete inhibition of Na^+-K^+-ATPase at higher drug concentrations is associated with lysis of the membrane. Any complex membrane function like ion transport or oxidative phosphorylation will therefore be inhibited at these drug concentrations. At these concentrations psychotropic drugs may also have direct actions on enzyme protein in the absence of lipid membrane material (CHOWDHURY *et al.* 1969).

Acknowledgments

The technical assistance of Mrs. Marit Haug and Miss Lilly Skutle is gratefully acknowledged.

We wish to express our thanks to Norfarma and Sandoz for the generous supply of drugs for this investigation.

REFERENCES

Chowdhury, A. K., H. Rogers, A. Skinner, R. G. Spector & D. C. Watts: The influence of psychotropic drugs on aldolase, mitochondrial malic dehydrogenase and Mg^{++} Na^{+} K^{+} adenosine triphosphatase. *Brit. J. Pharmacol.* 1969, **37**, 459–467.

Davis, P. W. & T. M. Brody: Inhibition of Na^{+}/K^{+}-activated adenosine triphosphatase activity in rat brain by substituted phenothiazines. *Biochem. Pharmacol.* 1966, **15**, 703–710.

Fiske, C. H. & J. Subbarow: Colorimetric determination of phosphorus. *J. biol. Chem.* 1925, **66**, 375–400.

Freeman, A. R. & M. A. Spirtes: Effects of chlorpromazine on biological membranes II. *Biochem. Pharmacol.* 1963, **12**, 47–53.

Greig, M. E. & A. J. Gibbons: Possible mechanism of action by which phenothiazine derivatives preserve stored blood. *Amer. J. Physiol.* 1955, **181**, 313–318.

Halpern, B. N, B. Dreyfus & G. Bourdon: Influence favorable sur la conservation du sang des dérivés de la phenothiazine. *Presse med.* 1958, **58**, 1151–1153.

Israel, J. & I. Salazar: Inhibition of brain microsomal adenosine triphosphatases by general depressants. *Arch. Biochem. Biophys.* 1967, **122**, 310–317.

Kraus, P. & Z. Šimáně: The influence of perathiepine and chlorpromazine on some enzyme reactions in rat brain preparations. *Experientia* 1967, **23**, 90–91.

Landmark, K.: The effect of thioridazine and promazine in reducing the potassium loss from isolated perfused rat hearts. *Acta pharmacol. et toxicol.* 1971, **29**, 49–59.

Landmark, K., A. Glomstein & I. Øye: The effect of thioridazine and promazine on the isolated contracting rat heart. *Acta pharmacol. et toxicol.* 1969, **27**, 173–182.

Langslet, A.: Cardiac effects of antiparkinson drugs, local anesthetics, and tranquilizers. *European J. Pharmacol.* 1970, **9**, 269–275.

Nakao, T., K. Nagano, K. Adachi & M. Nakao: Separation of two adenosine triphosphatases from erythrocyte membrane. *Biochem. Biophys. Res. Commun.* 1963, **13**, 444–448.

Pauling, L.: A molecular theory of general anesthesia. *Science* 1961, **134**, 15–21.

Post, R. L., C R. Merrit, C. R. Kinsolving & C. D. Albright: Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. *J. biol. Chem.* 1960, **235**, 1796–1802.

Robinson, J. D., J. Lowinger & B. Bettinger: Chlorpromazine: differential effects on membrane-bound enzymes from rat brain. *Biochem. Pharmacol.* 1968, **17**, 1113–1116.

Seeman, P. & J. Weinstein: I. Erythrocyte membrane stabilization by tranquilizers and antihistamines. *Biochem. Pharmacol.* 1966, **15**, 1737–1752.

Shanes, A. M.: Electrochemical aspects of physiological and pharmacological action in excitable cells. *Pharmacol. Rev.* 1958, **10**, 59–164.

Skou, J. C.: The influence of some cations on the adenosine triphosphatase from peripheral nerves. *Biochim. biophys. Acta* 1957, **23**, 394–401.

Skou, J. C.: Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. *Physiol. Rev.* 1965, 45, 596–617.

Trevor, A. J. & J. T. Cummins: Properties of sodium- and potassium activated adenosine triphosphatases of rat brain – effect of cyclopropane and other agents modifying enzyme activity. *Biochem. Pharmacol.* 1969, 18, 1157–1167.

Ueda, I. & W. Mietani: Microsomal ATPase of rabbit brain and effects of general anesthetics. *Biochem. Pharmacol.* 1967, 16, 1370–1374.

Whittam, R.: The assymetrical stimulation of a membrane adenosine triphosphatase in relation to active cation transport. *Biochem. J.* 1962, 84, 110–118.

Acta pharmacol. et toxicol. 1971, 29, 9–19.

From the Department of Pharmacology and the III Department of Pathology,
University of Helsinki, Finland

Hepatotoxicity of Halothane Metabolites in Vivo and Inhibition of Fibroblast Growth in Vitro

By

Per H. Rosenberg and Torsten Wahlström

(Received January 5, 1970)

Abstract: Following single intraperitoneal doses the halothane metabolites, trifluoroethanol (TFE) and trifluoroacetaldehyde hydrate (TFAlH), produced a marked dose-dependent accumulation of fat in mouse liver, the maximal effect occuring at about 24 hours after administration. Slight fatty changes already occurred with non-lethal doses, but repeated administration did not, however, cause necrosis of the liver cells. Electron microscopy suggested that part of the fat was composed of phospholipids, which are known to accumulate during energy loss. The glycogen granules almost completely disappeared in the liver, this being also confirmed by chemical determination. Trifluoroacetic acid (TFAA) not only caused slight fat accumulation, but also an increase in liver glycogen. In human fibroblast cell cultures TFAlH, even in small concentrations, and TFAA in high concentrations, inhibited cell growth. TFE did not affect the growth of the fibroblasts, probably because no enzymatic system is available in these cells to metabolize TFE to the toxic aldehyde. Two mechanisms may be responsible for the hepatotoxicity of TFE and TFAlH: a) formation of phospholipids due to energy requirement and b) accumulation of liver triglycerides due to blocking of e. g. transport enzymes.

Key-words: Halothane – hepatotoxicity – anaesthesia.

Halothane is believed to have produced severe hepatic necrosis in some patients (NATIONAL ACADEMY OF SCIENCES 1966). Whether this has been caused by a hypersensitivity reaction or by a direct hepatotoxic effect, has been widely discussed (e. g. KLATSKIN 1968).

About 17 % of the halothane absorbed is metabolized in man (REHDER *et al.* 1967). The main urinary metabolite is trifluoroacetic acid (TFAA), which is excreted for about two weeks after a halothane anaesthesia. TFAA is formed from halothane, probably via trifluoroethanol (TFE) and trifluoroacetaldehyde (VAN DYKE & CHENOWETH 1965). TFE, which is also a metabolite of another anaesthetic, fluroxene (BLAKE *et al.* 1967), is the most toxic of the metabolites (AIRAKSINEN & TAMMISTO 1968) and it is possible

that the toxic aldehyde formed from TFE, is responsible for the toxic actions (Airaksinen *et al.* 1970).

In mouse liver TFE and trifluoroacetaldehyde hydrate (TFAIH) have been found to inhibit anaerobic glycolysis *in vivo* and *in vitro* and TFE to decrease the ATP/ADP ratio *in vivo* probably resulting in defective energy production (Rosenberg *et al.* 1970).

During anaesthesia, halothane accumulates slowly in the liver (Duncan & Raventós 1959), although more slowly than e. g. in the fat, and the amount of the metabolites formed may therefore rise to a toxic level.

Material and Methods

The effect of trifluoroethanol (TFE, Fluka AG, Buchs SG), trifluoroacetaldehyde hydrate (TFAIH, Pierce Chem. Co., Rockford, Illinois), trifluoroacetic acid sodium (TFAA, Schuchardt, München) and halothane (Hoechst AG, Frankfurt M) was investigated *in vivo* and *in vitro*.

Table 1.

Histopathological changes in mouse liver caused by halothane and its metabolites after *intraperitoneal administration. The classification of the degree of injury (+ to +++)* is explained in the text.

Drug and dosage	Time after administration	Degree of liver injury
TFE 160 mg/kg	24 hrs	++
TFE 250 mg/kg	8 hrs	+
TFE 250 mg/kg	16 hrs	++
TFE 250 mg/kg	24 hrs	++
TFE 250 mg/kg	48 hrs	+
TFE 600 mg/kg	8 hrs	++
TFE 600 mg/kg	16 hrs	+++
TFE 100 mg/kg every 2nd day	14 days	++
TFAIH 300 mg/kg	24 hrs	+
TFAIH 700 mg/kg	12 hrs	+
TFAIH 700 mg/kg	24 hrs	++
TFAIH 1000 mg/kg	8 hrs	++
TFAIH 1000 mg/kg	16 hrs	+++
TFAIH 300 mg/kg every 2nd day	14 days	++
TFAA 1000 mg/kg	24 hrs	+
TFAA 2000 mg/kg	12 hrs	++
TFAA 2000 mg/kg	24 hrs	++
TFAA 2000 mg/kg every 2nd day	14 days	++
Halothane 1870 mg/kg	24 hrs	++

For the histological studies Swiss male mice weighing 17–20 grams were used. The drugs were injected intraperitoneally in different doses, and the animals, five in each series, were sacrificed at different intervals as shown in table 1. The livers were weighed and together with the heart and the kidneys fixed in formaline. Histological sections stained with haematoxylin-eosin (HE) were prepared for examination by light microscope. In some of the animals total and differential leukocyte counts were made.

Samples of the livers were also taken for electron microscopy. The fixation was performed with 2.5 % glutaraldehyde and post fixation with 1 % osmium tetroxide. The specimens were dried in a rising series of ethyl alcohol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate.

The glycogen content of the livers was determined according to VAN DER VIES (1954).

The cells used in the *in vitro* experiment were normal human skin fibroblasts in their second passage *in vitro*. Approximately 5×10^6 cells were plated in 50 mm Petri dishes with 5 ml of F-10 nutrient mixture (Gibco, Grand Island, New York) supplemented with 15 % heat-inactivated newborn calf serum and antibiotics (50 μ/ml aureomycin, Lederle). These were allowed to become attached overnight before the drugs to be tested in the amounts given in fig. 3 were added. The cultivation was interrupted three days later, and the cultures fixed with methanol for ten minutes and stained by the method of May-Grünwald-Giemsa. Four dishes were used for each drug and each concentration, and the cells were counted along two diameters, 2 mm across, and crossing each other at right angles.

Results

Histopathological changes in vivo.

No changes were observed in the histology of the heart or the kidneys of the animals treated with TFE, TFAlH, TFAA and halothane, as compared to the same organs of control mice. Marked histological changes were, however, seen in the livers of the test animals (fig. 1). In table 1, the severity of the liver changes has been graded from + to +++. One + represents a cloudy swelling of the hepatocytes, together with a slight fat accumulation, not yet seen as vacuolization of the cytoplasm, but rather presenting a coarse appearance of the cytoplasm. At the next stage of severity, scored as two +, vacuolization of the hepatocytes and lymphocytic depletion of Glisson's triads were seen. Although the lymphocytes completely disappeared, there was no significant variation in the peripheral leukocyte counts. In the most severely damaged livers, compression of the sinusoids, congestion of the portal vein branches as well as of the central veins, together with the features described above, were observed and scored as three +. No signs of biliary stasis were seen in any of the livers, and small haemorrhages found in some cases were considered to be artefacts. The weights of the livers did not change significantly.

The electron microscopic study of the livers graded in light microscopy

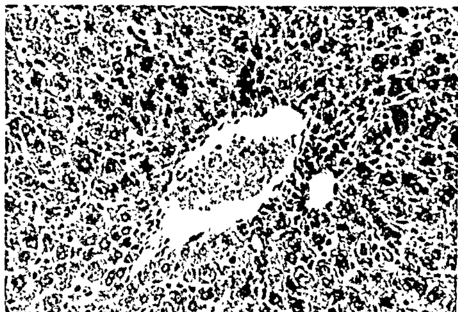


Fig. 1a. Control liver showing clearly visible sinusoids with conspicuous leukocytes. The portal vein branch is moderately filled with blood and the vicinity of the bile capillary is infiltrated by lymphocytes. Staining HE. Original magnification × 250.

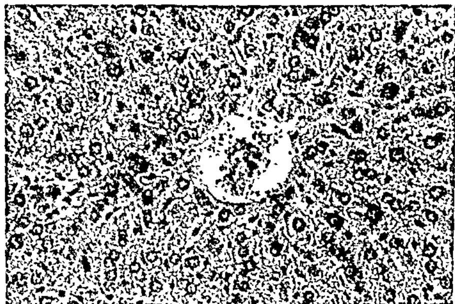


Fig. 1b. Liver from a mouse injected with TFE 250 mg/kg 8 hrs before sacrifice. The sinusoids are compressed and the cytoplasm of the hepatocytes has a coarse appearance (+ in table 1) Staining HE Original magnification × 250.

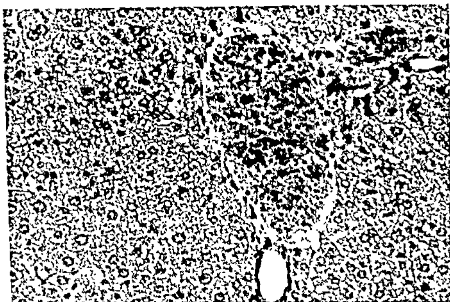


Fig. 1c. Liver from a mouse injected with TFAlH 700 mg/kg 24 hrs before sacrifice. Vacuoles of varying sizes are seen in the cytoplasm of the hepatocytes, the sinusoids are completely compressed, the lymphocytic infiltration normally seen next to the bile capillary has disappeared, and the portal vein branch is congested. (++ in table 1). Staining HE. Original magnification × 250.

Fig. 1d. Liver from a mouse injected with TFE 600 mg/kg 16 hrs before sacrifice. The liver cells contain large fat vacuoles and their cytoplasm is compressed to a thin rim at the periphery of the cells. (+++ in table 1). Staining HE. Original magnification × 250.



Fig. 2a An electron micrograph of a normal mouse liver. The glycogen granules are prominent. Magnification × 20000.

with ++ and +++ suggested that the glycogen granules had almost completely disappeared (fig. 2). This was confirmed by chemical determination of glycogen in the same livers (table 2). Although TFAA (2000 mg/kg) slightly injured the livers 24 hours after the administration, the content of glycogen rather tended to increase, in contrast to the effect of halothane and of the other metabolites.

Fat accumulation was the other prominent feature in the electron micrographs, and some of the vacuoles contained myelin figures (fig. 2b). The mitochondria showed no definite signs of injury.

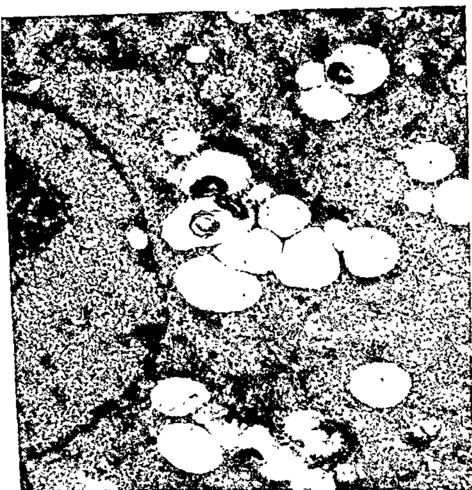


Fig. 2b. An electron micrograph of the liver from a mouse treated with TFAIH 700 mg/kg 24 hrs before sacrifice. The glycogen granules seem to have almost completely disappeared and fat vacuoles dominate the picture. Inside some of the vacuoles myelin figures can be seen. Magnification × 20000.

As seen in table 1, the extent of liver injury was dose-dependent, and the maximal effect with TFE and TFAIH occurred at about 24 hours after administration. There were already fatty changes with non-lethal doses of TFE (160 mg/kg) and TFAIH (300 mg/kg), which were reversible. Necrosis of the liver cells could not be detected even after multiple doses of TFE, TFAIH and TFAA (administered every second day during two weeks).

Table 2.

Effect of TFE, TFAIH, TFAA and halothane on the liver glycogen in mice 24 hrs after intraperitoneal administration. Six determinations for each value.

	Degree of liver injury	Glycogen mg/100 g (± S.E.M.)
Control	—	205.9 ± 28.8
TFE 250 mg/kg	++	16.3 ± 7.9
TFE 600 mg/kg	+++	1.6 ± 1.0
TFAIH 700 mg/kg	++	3.2 ± 1.1
TFAIH 1000 mg/kg	+++	1.9 ± 0.8
TFAA 2000 mg/kg	++	301.6 ± 25.5
Halothane 1870 mg/kg	++	1.3 ± 0.7

Inhibition of fibroblast growth in vitro.

TFE in concentrations up to 50 mM did not have any inhibitory effect on the growth of the fibroblasts as compared to the controls (fig. 3). Neither did it cause any morphological changes in the test cells. On the other hand, TFAIH, even in low concentrations, and TFAA, in high concentrations, markedly inhibited the multiplication of the cells, as judged by their decreasing number in the dishes with increasing concentrations of the drugs. Many of the cells in these dishes showed a pycnotic nucleus and a pale cytoplasm, suggesting an inhibition of the metabolism of the cells.

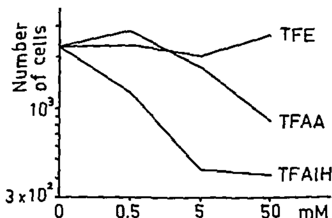


Fig. 3. Effect of TFE, TFAIH and TFAA on human fibroblast growth *in vitro*. The cultures were interrupted three days after administration of the drugs. The cells were then counted as described in the text. Four dishes for each concentration.

Discussion

TFAA has been shown to cause enlargement of the mouse liver and slight functional disturbances, but no fat accumulation when administered in the drinking water for 4–5 days (SCHIMASSEK *et al.* 1966). Contrary to the reports of BLAKE *et al.* (1969) TFE and TFAIH markedly injured the livers of mice in our studies. Fat accumulated even after single non-lethal doses and the vacuolization and destruction of the lobar structure by lethal doses might be sufficient to explain the cause of death. The myelin figures of the fat vacuoles indicate that some of the fat was composed of phospholipids (SCHOLLER 1966). The same has been observed after prolonged halothane anaesthesia (SCHOLLER 1966). This means that fatty acids from the fat depots combine with glycerophosphates for the formation of phospholipids (BRODIE & MAICKEL 1963). The inhibition of energy production by TFE and TFAIH (ROSENBERG *et al.* 1970) therefore leads to an energy requirement and an accumulation of external fatty acids in the liver.

On the other hand, we have found a significant decrease of reduced glutathione in the liver and erythrocytes (ROSENBERG unpublished), suggesting an inhibition of enzymes with active thiol groups (AIRAKSINEN *et al.* 1970). This would thus lead to a defective utilization and transport of triglycerides in the liver and to an accumulation of fat, i. e. the hepatotoxic sign of injury (REES & SHOTLANDER 1963). Chloroform and carbon tetrachloride are believed to act by this mechanism in the production of fatty liver (SCHOLLER 1966). It is therefore probable that both mechanisms are effective in causing the liver injury due to halothane metabolites.

Glycogen, which decreases in the liver when general hepatotoxins are administered (BASSI 1960), almost totally disappeared from the liver cells after treatment with TFE, TFAIH and halothane. In this case glycogenolysis was probably stimulated by energy loss, but it might also have been stimulated by anaesthesia, as has been shown by VIRTUE *et al.* (1958).

It has been concluded that halothane is not necrogenic in experimental animals (NORRIS *et al.* 1963; BLOXAM 1967) as are e. g. chloroform and carbon tetrachloride, and the slight hepatic damage sometimes seen is probably due to hepatic hypoxia (GALINDO *et al.* 1966). Administration of the anaesthetic directly into the stomach of mice caused only fatty changes and so did multiple exposures (JONES *et al.* 1958). The present study suggests that neither of the halothane metabolites cause necrosis of the liver cells.

In our studies TFAIH inhibited the growth of human fibroblasts *in vitro* in concentrations which did not greatly exceed the amount of the drug which would be expected to accumulate in the liver and the kidneys after administration of LD50 doses (AIRAKSINEN & TAMMISTO 1968). The fact that TFE had no effect on the fibroblasts seems to indicate that there is no enzymatic

Table 2.

Effect of TFE, TFAlH, TFAA and halothane on the liver glycogen in mice 24 hrs after intraperitoneal administration. Six determinations for each value.

	Degree of liver injury	Glycogen mg/100 g (± S.E.M.)
Control	–	205.9 ± 28.8
TFE 250 mg/kg	++	*16.3 ± 7.9*
TFE 600 mg/kg	+++	*1.6 ± 1.0*
TFAlH 700 mg/kg	++	3.2 ± 1.1
TFAlH 1000 mg/kg	+++	1.9 ± 0.8
TFAA 2000 mg/kg	++	301.6 ± 25.5
Halothane 1870 mg/kg	++	1.3 ± 0.7

Inhibition of fibroblast growth in vitro.

TFE in concentrations up to 50 mM did not have any inhibitory effect on the growth of the fibroblasts as compared to the controls (fig. 3). Neither did it cause any morphological changes in the test cells. On the other hand, TFAlH, even in low concentrations, and TFAA, in high concentrations, markedly inhibited the multiplication of the cells, as judged by their decreasing number in the dishes with increasing concentrations of the drugs. Many of the cells in these dishes showed a pycnotic nucleus and a pale cytoplasm, suggesting an inhibition of the metabolism of the cells.

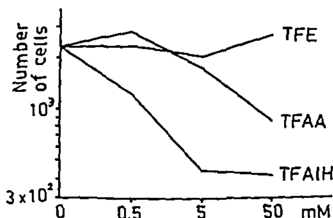


Fig. 3. Effect of TFE, TFAlH and TFAA on human fibroblast growth *in vitro*. The cultures were interrupted three days after administration of the drugs. The cells were then counted as described in the text. *Four dishes for each concentration.*

Fink, B. R. & G. E. Kenny: Effects of halothane on cell culture metabolism. In: *Toxicity of anesthetics*. Ed. B. R. Fink. The Williams & Wilkins Co., Baltimore 1968.
Galindo, A., G. F. Brindle & R. G. B. Gilbert: Hepatic circulation and hepatic function during anaesthesia and surgery. IV Halothane Anaesthesia. *Canad. Anaesth. Soc. J.* 1966, 13, 328–341.
Jones, W. M., G. Margolis & C. R. Stephen: Hepatotoxicity of inhalation anesthetic drugs. *Anesthesiology* 1958, 19, 715–723.
Klatskin, G.: Introduction: Mechanism of toxic and drug induced hepatic injury. In: *Toxicity of anesthetics*. Ed. B. R. Fink. The Williams & Wilkins Co., Baltimore 1968.
National Academy of Sciences: Summary of the national halothane study – Possible association between halothane anesthesia and postoperative hepatic necrosis. *J. Amer. med Ass.* 1966, 197, 775–788.
Norris, F. H., P. H. Geisler, W. L. Pritchard & K. D. Hall: Lack of necrosis in dog liver after anaesthesia with halothane. *Arch. int. Pharmacodyn.* 1963, 145, 405–412.
Rees, K. R. & V. L. Shotlander: Fat accumulation in acute liver injury. *Ann. N. Y. Acad Sci.* 1963, 104, 905–915.
Rehder, K., J. Forbes, H. Alter, O. Hessler & O. Stier: Halothane biotransformation in man: A quantitative study. *Anesthesiology* 1967, 28, 711–715.
Rosenberg, P. H., M. M. Airaksinen & T. Tammisto: Inhibition of energy production by halothane metabolites. *Acta pharmacol. et toxicol.* 1970, 28, 327–333.
Schimassek, H., J. Helms, W. Kunz & A. Stier: Lebervergrössung unter Trifluoressigsäure, Spaltprodukt des Halothan. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* 1966, 255, 67–68.
Scholler, K. L.: Elektronmikroskopische Untersuchungen an Leberzellen der Ratte nach Narkosen mit verschiedenen Inhalationsnarkotika. *Anaesthesist* 1966, 15, 145–148.
Van Dyke, R. A. & M. B. Chenoweth: Metabolism of volatile anesthetics. *Anesthesiology* 1965, 26, 348–357.
Vies, J. van der: Two methods for the determination of glycogen in liver. *Biochem. J.* 1954, 57, 410–416.
Virtue, R. W., K. W. Payne, L. J. Caranna, G. S. Gordon & R. R. Rember: Observations during experimental and clinical use of fluothane. *Anesthesiology* 1958, 19, 478–487.

Acta pharmacol. et toxicol. 1971, 29, 20–32.

From the Psychiatric Research Center, Ulleråker Hospital,
University of Uppsala, Uppsala, Sweden

Displacement of Brain and Heart Noradrenaline by *p*-Hydroxynorephedrine after Administration of *p*-Hydroxyamphetamine

By

Tommy Lewander

(Received May 19, 1970)

Abstract: After the administration of *p*-hydroxyamphetamine 20 mg/kg intraperitoneally, there was a 50 per cent decrease in the brain noradrenaline (NA) level and a 70 per cent decrease in the heart NA level at 4–8 hours after the injection. Control levels of brain and heart NA were reached at 96–144 hours after a single dose of the drug. Repeated injections of *p*-hydroxyamphetamine, 20 mg/kg twice daily for 3 days, caused a depletion of brain and heart NA to about 20 per cent of the control levels. The brain dopamine (DA) level was reduced to 85 per cent of the control level at 1 hour after 20 mg/kg of the drug and to 76 per cent after 40 mg/kg. After the repeated injections of *p*-OH-A, the brain DA was reduced to 70 per cent of the control level. Radioactively labelled *p*-hydroxynorephedrine was isolated from both brain and heart tissue extracts after the administration of *p*-hydroxyamphetamine-^{3}H. Parahydroxynorephedrine-^{3}H remained in the brain and the heart as long as the NA levels were decreased. The NA deficit in the brain and heart corresponded approximately to the amounts of *p*-hydroxynorephedrine-^{3}H present in the tissues. In reserpine pretreated rats the amounts of *p*-hydroxynorephedrine-^{3}H formed in the brain and heart tissues were reduced, indicating that *p*-hydroxynorephedrine is bound by a reserpine sensitive storage mechanism. Administration of *p*-hydroxyamphetamine or amphetamine accelerated the disappearance of labelled *p*-hydroxynorephedrine from the brain and the heart.

Key-words: Rat – brain – catecholamines – sympathomimetics.

In rats, parahydroxyamphetamine is the major metabolite of amphetamine (Axelrod 1954; Alleva 1963; Dring *et al.* 1966; Ellison *et al.* 1966). It has been demonstrated that the intraperitoneal administration of *p*-hydroxyamphetamine causes a decrease in brain and heart NA[1] (Maître & Brunner

---

1) Abbreviations used: NA = noradrenaline, DA = dopamine, CA = catecholamine(s).

1967; LEWANDER 1968) and it was concluded (LEWANDER 1968) that *p*-hydroxyamphetamine might contribute to the changes in tissue CA levels caused by amphetamine. This conclusion was supported by the previous finding of *p*-hydroxynorephedrine (or α-methyloctopamine) as a metabolite of amphetamine in peripheral NA neurons (GOLDSTEIN & ANAGNOSTE 1965; THOENEN *et al.* 1966). In addition, *p*-hydroxynorephedrine has been isolated from peripheral adrenergically innervated organs after the administration of *p*-hydroxyamphetamine (KOPIN 1965). These facts prompted us to study the effects of *p*-hydroxyamphetamine on brain and heart CA in relation to the presence of *p*-hydroxyamphetamine and *p*-hydroxynorephedrine in these tissues.

Recently, it was independently reported by GROPPETTI & COSTA (1969), COSTA & GROPPETTI (1970), BRODIE & GESSA (1970) and LEWANDER (1970), that *p*-hydroxynorephedrine is also found in the CNS after the administration of amphetamine.

Material and Methods

Male Sprague-Dawley rats, 200 g body weight, were used. The animals were kept in individual cages for at least 24 hours before and during the experiments. Food and water was given *ad libitum*.

After treatment with racemic *p*-hydroxyamphetamine-HBr (Smith, Kline and French) dissolved in saline and in doses given under results, the rats were decapitated under light chloroform anaesthesia and the brains and hearts were rapidly dissected out and homogenized in 0.4 N perchloric acid. CA in the tissue extracts were adsorbed on alumina (0.6 g in glass columns). NA in the acetic acid eluate was determined according to CHANG (1964) and DA according to CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962).

^{3}H-dl-*p*-hydroxyamphetamine, generally labelled, was obtained by acid catalytical exchange (AB Atomenergi, Studsvik, Sweden). Before use, a radiochemical purity of > 99 % was ascertained by isolation of the labelled *p*-hydroxyamphetamine on an ion-exchange column (Dowex 50 W X-4, Na^+-form, 0.4 × 5 cm, run as described below for Amberlite columns) and subsequent checking by paper and thin-layer chromatography (see below). The ^{3}H-*p*-hydroxyamphetamine was added to the unlabelled compound to give a specific activity of 0.57 mci/mmole. Each rat received approximately 100 μci = 4 mg of the drug. Labelled *p*-hydroxyamphetamine and *p*-hydroxynorephedrine in the perchloric acid extracts (set to pH 4.5 by addition of 2 M-KOH) of brain and heart tissues were separated on Amberlite CG 120, type II, Na^+-form, ion-exchange columns (0.4 × 5 cm). The two substances appeared in the 1 M-HCl-eluate as shown in fig. 3. For quantitative determinations the eluate was divided into 3 portions which were collected separately: 1. The first 2 ml were discarded, 2. 15 ml containing *p*-hydroxynorephedrine and 3. 30 ml containing *p*-hydroxyamphetamine. The second and third portions were reduced to dryness, redissolved in methanol and transferred to counting vials. After the addition of 14 ml of a scintillation mixture (0.1 g POPOP and 4.0 g of PPO per litre of toluene) the vials were counted in a Packard Tri-Carb scintillation spectrometer. Quenching was monitored by the external standard

Recoveries were 92 ± 3.9 % (mean ± S. D.) for *p*-hydroxynorephedrine and 78–80 % for *p*-hydroxyamphetamine as determined at 285/335 nm in 1 M-HCl (uncorrected) in an Aminco-Bowman spectrophotofluorometer.

Parahydroxynorephedrine in the second portion and *p*-hydroxyamphetamine in the third portion of the ion exchange column eluate were identified by paper chromatography according to ELLISON *et al.* (1966) and by thinlayer chromatography on ChromAR® (Malinckrodt) silica gel sheets developed in benzene-pyridine-acetic acid (30/1/10). Authentic amines were visualized by staining with ninhydrine. Radioactivity in 1 cm segments of the strips of the paper or silica gel sheet was counted as described above. Details of the above procedures are described elsewhere (LEWANDER 1971).

Tissue levels of CA, *p*-hydroxynorephedrine and *p*-hydroxyamphetamine refer to the respective bases.

Comparison of the data was made by use of Student's t-test.

Results

Changes in tissue levels of CA after p-hydroxyamphetamine.

It is shown in fig. 1 that brain and heart NA are maximally depressed at 4 and 8 hours respectively after the administration of 20 mg/kg intraperitoneally of *p*-hydroxyamphetamine and that replenishment of the NA stores was not reached until 6 days later. Brain DA was unchanged except for a decrease of 25 % ($P < 0.001$) at 1 hour (data not shown) and of 14 % ($P < 0.05$) at 8 hours after the drug was administered.

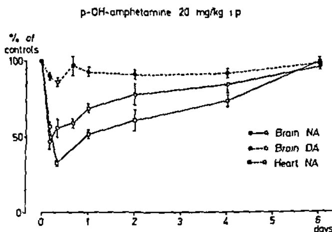


Fig. 1. Time-response curves of changes in brain noradrenaline (NA), brain dopamine (DA) and heart NA in the rat induced by a single dose of *p*-hydroxyamphetamine (20 mg/kg intraperitoneally) injected at zero time. Each point represent the mean (± S. E. M.) of 4–6 determinations. Open symbols indicate statistically significant ($P < 0.01$ or less) differences from the control level. Absolute values for: brain NA = 0.39 ± 0.01 µg/g, brain DA = 0.84 ± 0.03 µg/g, heart NA = 0.77 ± 0.02 µg/g.

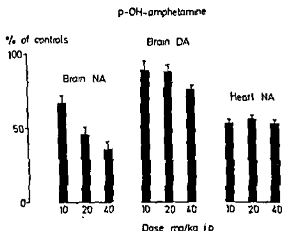


Fig. 2. Dose response relationship between administered *p*-hydroxyamphetamine and the levels of brain noradrenaline (NA), brain dopamine (DA) and heart NA in the rat, measured 4 hours after the injection of the drug. Columns and vertical bars represent means + S. E. M. of 5–6 observations. The absolute catecholamine levels were: Brain NA = 0.39 ± 0.01 µg/g, brain DA = 0.84 ± 0.02 µg/g and heart NA = 0.77 ± 0.02 µg/g.

Brain and heart NA and brain DA levels were determined at 4 hours *after 3 different doses of p-hydroxyamphetamine injected intraperitoneally*. It is seen (fig. 2) that there is a dose-response relationship with regard to the decrease in brain NA. The heart NA was equally depressed after all 3 doses. The brain DA was decreased by 24 % ($P < 0.001$) after the administration of the highest dose of the drug.

Table 1.

The effect of a single injection (20 mg/kg intraperitoneally) and repeated injections (20 mg/kg twice daily for three days) of *p*-hydroxyamphetamine (*p*-OH-A) on the levels of brain dopamine (DA), brain noradrenaline (NA) and heart NA in the rat expressed as percentages of control levels (means ± S. E. M. of 5–6 observations). The animals were sacrificed 4 hours after the last injection. The absolute control levels are the same as given in fig. 2.

Treatment	Brain		Heart
	DA	NA	NA
saline ...	100 ± 2.4	100 ± 3.1	100 ± 3.2
p-OH-A, single ...	88 ± 5.5	46 ± 2.1***	56 ± 2.4***
p-OH-A, repeated ...	70 ± 1.8***	21 ± 7.7***	18 ± 1.0***

*** Difference versus control $P < 0.001$.

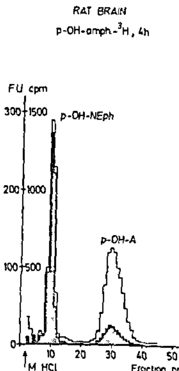


Fig. 3. Ion-exchange chromatography of a brain extract from a single rat injected with *p*-hydroxyamphetamine-^{3}H 4 hours previously. Solid line: Native fluorescence (285/335 nm) in fluorescence units (F. U.). Broken line (= shaded area): Radioactivity in cpm. Abbreviations: *p*-OH-NEph = *p*-hydroxynorephedrine, *p*-OH-A = *p*-hydroxyamphetamine.

In a third experiment the effect of a single injection of *p*-hydroxyamphetamine, 20 mg/kg intraperitoneally was compared with the effect of repeated injections, 20 mg/kg twice daily for 3 days, on brain and heart CA levels at 4 hours after the last injection (table 1). Both the brain and heart NA were decreased by 50 % after one injection and by 80 % after repeated injections. The brain DA was significantly decreased ($P < 0.001$) only after repeated injections of *p*-hydroxyamphetamine.

Identification and quantitative determination of p-hydroxynorephedrine in brain and heart tissues.

At 4 hours after the intraperitoneal administration of *p*-hydroxyamphetamine-^{3}H, 0.02 % of the injected dose was found in the brain and the heart tissue extracts. After the addition of authentic *p*-hydroxyamphetamine and *p*-hydroxynorephedrine to the tissue extract the 2 compounds were separated by ion-exchange chromatography. It was found that radioactivity in the brain

extract followed both the *p*-hydroxynorephedrine and the *p*-hydroxyamphetamine peak (fig. 3). The fractions containing *p*-hydroxynorephedrine (no. 5–15) were pooled, concentrated and further chromatographed on paper or thin-layer as described in "Methods." The radioactive material cochromatographed with authentic *p*-hydroxynorephedrine in both systems.

The same results were obtained with heart tissue extracts from rats given *p*-hydroxyamphetamine-^{3}H.

About 50 % of the total radioactivity in the brain extract and 60 % in the heart extract was found in the effluent and washings from the ion-exchange columns. No attempts were made to identify this radioactive material.

The amounts of *p*-hydroxyamphetamine and *p*-hydroxynorephedrine in the brain and heart were determined at different intervals after administration of *p*-hydroxyamphetamine-^{3}H (fig. 4). At 4 hours after the injection, peak levels of *p*-hydroxynorephedrine were found in the brain and in the heart at 8 hours. Measurable amounts of *p*-hydroxynorephedrine were found both in the brain and heart tissue for 6 days. Parahydroxyamphetamine was present in the tissues during the first 24 hours after administration of the drug but disappeared during the following day.

Relationship between depletion of NA and occurrence of p-hydroxynorephedrine in brain and heart after p-hydroxyamphetamine.

According to fig. 5 the NA deficit caused by *p*-hydroxyamphetamine is almost stoichiometrically covered by its metabolite *p*-hydroxynorephedrine in both the brain and heart, at least during the first 48 hours after the

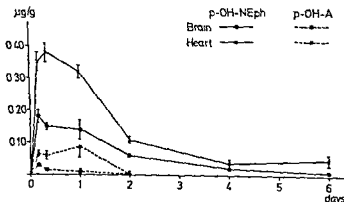


Fig. 4. Time course of the presence of *p*-hydroxyamphetamine (*p*-OH-A) and *p*-hydroxynorephedrine (*p*-OH-NEph) in rat brain and heart tissue extracts respectively after a single injection of *p*-OH-A-^{3}H (20 mg/kg intraperitoneally).

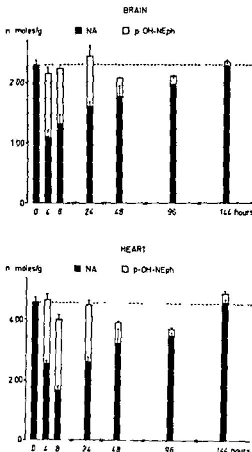


Fig. 5. Remaining stores of noradrenaline (NA, black columns) in rat brain (above) and heart (below) tissues at different times after the administration of *p*-hydroxyamphetamine (20 mg/kg intraperitoneally). White columns, on top of the black columns, represent the amounts of *p*-hydroxynorephedrine (*p*-OH-NEph) present in the tissues at the respective times. Broken lines: Control levels of NA. Columns and vertical bars represent means + S. E. M. of 4–6 observations.

p-hydroxyamphetamine injection. The NA and *p*-hydroxynorephedrine values, corrected for recoveries, were obtained from 2 separate groups of rats (cf. fig. 1 and fig. 4).

Effect of reserpine pretreatment on p-hydroxynorephedrine levels in brain and heart.

Four rats were given reserpine, 5 mg/kg intraperitoneally, and 4 other rats saline 8 hours before sacrifice. All the rats received *p*-hydroxyamphet-

amine-^{3}H, 20 mg/kg intraperitoneally, 4 hours before sacrifice. The determination of *p*-hydroxynorephedrine formed from *p*-hydroxyamphetamine showed (table 2) that the brains and hearts of the reserpine treated rats contained only 11 % ($P < 0.001$) and 4 % ($P < 0.001$) respectively of the control *p*-hydroxynorephedrine levels.

Effects of p-hydroxyamphetamine and amphetamine on p-hydroxynorephedrine levels in brain and heart.

Parahydroxynorephedrine-^{3}H was incorporated into the brain and heart by the administration of *p*-hydroxyamphetamine-^{3}H, 20 mg/kg intraperitoneally, 20–21 hours before the start of the experiment. The animals were divided into 4 groups of 4–5 rats. In the first experiment 2 groups received unlabelled *p*-hydroxyamphetamine, 20 mg/kg intraperitoneally (= 0.086 nmole of the base) and saline respectively and were sacrificed 4 hours later. In a second experiment the 2 other groups were given dl-amphetamine-SO_4, 20 mg/kg intraperitoneally (= 0.110 nmole of the base) or saline and were sacrificed 3 hours after the last injection.

As shown in table 3 *p*-hydroxyamphetamine caused a decrease in both the brain and heart *p*-hydroxynorephedrine-^{3}H levels to 70 % ($P < 0.001$) and 14 % ($P < 0.001$) of the control values.

Administration of amphetamine was also followed by a decrease in the brain and heart p-hydroxynorephedrine-^{3}H levels to 90 % ($P < 0.05$) and 26 % ($P < 0.001$) respectively as compared with the controls.

Table 2.

Content of *p*-hydroxynorephedrine (p-OH-NEph) in rat brain and heart tissues 4 hours after the administration of *p*-hydroxyamphetamine to control rats (set to 100 %) and rats pretreated with reserpine, 5 mg/kg intraperitoneally 4 hours before the *p*-hydroxyamphetamine injection. *Figures represent means ± S. E. M. of 4 observations.*

Pretreatment	p-OH-NEph			
	Brain		Heart	
	ng/g	%	ng/g	%
Saline	160 ± 20	100	350 ± 60	100
Reserpine, 5 mg/kg i. p.	17 ± 10***	11	15 ± 8***	4

*** Difference versus saline $P < 0.001$.

Table 3.

Effect of *p*-hydroxyamphetamine and amphetamine on brain and heart tissue levels of *p*-hydroxynorephedrine. *Parahydroxynorephedrine*-^3H *was synthesized in vivo by* administration of *p*-hydroxyamphetamine-^3H, 20 mg/kg intraperitoneally, 24 hours before sacrifice. Cold *p*-hydroxyamphetamine, 20 mg/kg intraperitoneally, or dl-amphetamine-SO_4, *20 mg/kg intraperitoneally, was administered 4 or 3 hours respectively before* sacrifice. Figures represent means ± S. E. M. of 4–5 observations.

Treatment	*p*-OH-NEph			
	Brain		Heart	
	ng/g	%	ng/g	%
Expt. I:				
saline ..	81 ± 1	100	283 ± 16	100
p-hydroxyamph.	57 ± 1***	70	39 ± 8***	14
Expt. II:				
saline	97 ± 4	100	319 ± 24	100
dl-amph.	87 ± 1*	90	82 ± 6***	26

* Difference versus saline $P < 0.05$.
*** Difference versus saline $P < 0.001$.

Discussion

Like tyramine (CARLSSON & WALDECK 1963; FISCHER *et al.* 1964), *p*-hydroxyamphetamine has been shown to be β-hydroxylated in peripheral adrenergic neurons (KOPIN *et al.* 1965). The present results confirm these observations, which are extended to include the quantitative relationships between the presence of *p*-hydroxynorephedrine and the depletion of NA in the rat heart. Furthermore, both *p*-hydroxyamphetamine and its β-hydroxylated metabolite *p*-hydroxynorephedrine were identified in the brain tissue. The depletion of brain NA caused by *p*-hydroxyamphetamine (MAÎTRE & BRUNNER 1967; LEWANDER 1968) was found to be dose-dependent and to persist as long as *p*-hydroxynorephedrine was present in the brain tissue (about 96–144 hours).

Parahydroxyamphetamine was found both in the brain and heart tissues, but in smaller amounts and for a shorter time than its β-hydroxylated metabolite (cf. fig. 4). Parahydroxynorephedrine was present much longer with an apparant half-life of 22–24 hours in both brain and heart. This figure is in agreement with the $T_{1/2}$ of *p*-hydroxynorephedrine obtained by KOPIN *et al.* 1965 in the rat heart. The same relationship between *p*-hydroxyamphetamine

and *p*-hydroxynorephedrine in rat brain and heart after administration of amphetamine was reported by GROPPETTI & COSTA (1969).

Since the enzyme β-hydroxylase is present in NA neurons only and since the major part of the *p*-hydroxynorephedrine seems to be stored by a reserpine sensitive mechanism (table 2), there is reason to believe that *p*-hydroxynorephedrine, like octopamine after administration of tyramine (ANAGNOSTE & GOLDSTEIN 1967; CARLSSON & WALDECK 1963), and like other phenylethylamine derivatives (cf. KOPIN 1968) is bound to the NA storage granules as a false adrenergic transmitter. This is further supported by the present finding, that there is an almost stoichiometrical relationship between the amount of NA depleted from the brain and heart and the tissue levels of *p*-hydroxynorephedrine for at least the first 48 hours after the *p*-hydroxyamphetamine administration. A stoichiometric displacement of NA in the rat brain and heart caused by metaraminol after the administration of α-methylmetatyrosine has been reported previously (ANDÉN 1964).

From these observations it follows that *p*-hydroxyamphetamine actually passes the blood-brain barrier, which is generally considered as impermeable to this compound as well as to tyramine. The inability of tyramine to pass the blood brain barrier (cf. ANAGNOSTE & GOLDSTEIN 1967) may be due to its lability against MAO when penetrating the endothelium of brain capillaries (cf. BERTLER *et al.* 1966). According to the present results at most 0.012 % of the injected radioactivity was found per g brain tissue at 4 hours after the *p*-hydroxyamphetamine administration as opposed to 0.027 %/g heart tissue, indicating that there is a hindrance, although not complete, for the permeation of *p*-hydroxyamphetamine into the brain. In comparison a peak level of about 0.7 % of the dose (20 mg/kg intraperitoneally) is found per g brain at 30 minutes after an amphetamine injection (LEWANDER 1971).

The decrease in brain NA, but not in heart NA, was dependent on the dose of *p*-hydroxyamphetamine (fig. 2). Explanations for this discrepancy may be that the depletion of heart NA, is not maximal at the time interval (fig. 1) chosen in this experiment or that the NA released from other adrenergically innervated organs is taken up by the heart NA neurons in competition with *p*-hydroxyamphetamine (cf. IVERSEN 1966). A release of peripheral NA by *p*-hydroxyamphetamine is indicated by an increase in the urinary excretion of NA (LEWANDER 1968).

After a single injection of *p*-hydroxyamphetamine there was as much as a 50 % reduction in brain NA and a 70 % reduction in heart NA while after six consecutive injections of *p*-hydroxyamphetamine, only an additional 30 % in the brain and heart NA levels was depleted (table 1). This might indicate that part of the NA stores are refractory to the NA depleting action of *p*-hydroxyamphetamine. Another explanation, which is supported by the results shown in table 3, is that a second dose of *p*-hydroxyamphetamine in

addition to NA causes a displacement of p-hydroxynorephedrine, formed after the first injection.

So far there are no reports on the metabolism of *p*-hydroxynorephedrine in the brain. The α-methyl group protects both *p*-hydroxynorephedrine and *p*-hydroxyamphetamine from deamination by MAO. The small amount of *p*-hydroxynorephedrine-^{3}H released by *p*-hydroxyamphetamine from the brain in comparison to the heart (table 3) might possibly be due to the blood-brain barrier retarding the entrance of *p*-hydroxyamphetamine into the brain or delaying the disappearance of extragranular *p*-hydroxynorephedrine-^{3}H from the brain tissue.

The decrease in brain and heart levels of *p*-hydroxynorephedrine-^{3}H after amphetamine (table 3, expt. II) was smaller than that after *p*-hydroxyamphetamine. It cannot be concluded from this experiment whether amphetamine itself releases *p*-hydroxynorephedrine-^{3}H or not. A part at least of the missing *p-hydroxynorephedrine-*^{3}*H in the brain and heart might have been displaced* by *p*-hydroxyamphetamine formed from amphetamine in this experiment. Octopamine-^{14}C in the mouse heart was not released by amphetamine, while *p*-hydroxylated phenylethylamine derivatives were potent in this test (CARLSSON & WALDECK 1966). In the present experiment, heart *p*-hydroxynorephedrine-^{3}H might also have been released by an increased nerve impulse flow in the peripheral sympathetic nerves (THOENEN *et al.* 1966) caused by amphetamine.

The brain DA levels were only slightly and transiently decreased by the administration of *p*-hydroxyamphetamine. However, the changes observed (fig. 1, 2 & 3) indicate that *p*-hydroxyamphetamine has an action on brain DA. In support of this conclusion it has been reported that *p*-hydroxyamphetamine (50–200 μg) injected bilaterally into the caudate nuclei of the rat, induces an amphetamine-like stereotyped behavior (FOG cited by RANDRUP & MUNKVAD 1970). 3 hours after the intracisternal injection of 40 μg of *p*-hydroxyamphetamine, however, the brain DA was not affected (BREESE *et al.* 1970). Differences in dose, route of administration and interval between injection and sacrifice may explain this inconsistency.

Acknowledgements

The author is indebted to Mrs. Inger Jansson for skilful technical assistance. This work has been supported by the Swedish Medical Research Council (project Nos. B70-40X-1017-06B and B70-27P-2627-02) and by The Tri-Centennial Fund of the Bank of Sweden (project No. 150).

REFERENCES

Alleva, J. J.: Metabolism of tranylcypromine-^{14}C and dl-amphetamine-^{14}C in the rat. *J. med. Chem.* 1963, 6, 621–624.

Anagnoste, B. & M. Goldstein: The metabolism of tyramine-^{3}H in different regions of the CNS. *Life Sci.* 1967, 6, 1535–1540.

Andén, N.-E.: On the mechanism of noradrenaline depletion by α-methyl metatyrosine and metaraminol. *Acta pharmacol. et toxicol.* 1964, 21, 260–271.

Axelrod, J.: Studies on sympathomimetic amines. II. The biotransformation and physiological disposition of d-amphetamine, d-p-hydroxyamphetamine and d-methamphetamine. *J. Pharmacol. exp. Ther.* 1954, 114, 315–326.

Bertler, A., B. Falck, C. Owman & E. Rosengren: Properties of adrenergic tissues: The localization of mono-aminergic blood-brain barrier mechanisms. *Pharmacol. Rev.*, 1966, 18, 369–385

Breese, G. R., I. J. Kopin & V. K. Weise: Effects of amphetamine derivatives on brain dopamine and noradrenaline. *Brit. J. Pharmacol.* 1970, 38, 537–545.

Brodie, B. B., A. K. Cho & G. L. Gessa: Possible role of *p*-hydroxynorephedrine in the depletion of norephedrine induced by *d*-amphetamine and in tolerance of this drug. In: *International symposium on amphetamines and related compounds.* Ed.: E. Costa and S. Garattini. Raven Press. New York. 1970.

Carlsson, A. & B. Waldeck: A fluorimetric method for the determination of dopamine (3-hydroxytyramine). *Acta physiol. scand.* 1958, 44, 293–298.

Carlsson, A. & B. Waldeck: β-hydroxylation of tyramine *in vivo*. *Acta pharmacol. et toxicol* 1963, 20, 371–374.

Carlsson, A. & B. Waldeck: Structure-activity relationships for release of ^{14}C-octopamine from adrenergic nerves by phenylethylamines. *Acta pharmacol. et toxicol.* 1966, 24, 255–262.

Carlsson, A. & M. Lindqvist: *In vivo* decarboxylation of α-methyldopa and α-methyltyrosine. *Acta physiol scand* 1962, 54, 87–94.

Chang, C. C.: A sensitive method for spectrophotofluorimetric assay of catecholamines. *Int. J. Neuropharmacol.* 1964, 3, 643–649.

Costa, E. & A Groppetti: Biosynthesis and storage of catecholamines in tissues of rats injected with various doses of *d*-amphetamine. In: *International symposium on amphetamines and related compounds.* Ed.: E. Costa and S. Garattini. Raven Press, New York 1970.

Dring, L. G., R. L. Smith & R. T. Williams: The fate of amphetamine in man and other mammals. *J. Pharm Pharmacol.* 1966, 18, 402–404.

Ellison, T., L. Gutzait & E. J. van Loon: The comparative metabolism of d-amphetamine-^{14}C in the rat, dog and monkey. *J. Pharmacol. exp. Ther.* 1966, 152, 383–387.

Fischer, J E., J. Musacchio, I. J. Kopin & J. Axelrod: Effects of denervation on the uptake and β-hydroxylation of tyramine in the rat salivary gland. *Life Sci.* 1964, 3, 413–419.

Goldstein, M. & B. Anagnoste: The conversion *in vivo* of D-amphetamine to (+)-p-hydroxynorephedrine *Biochim Biophys. Acta* 1965, 107, 166–168.

Groppetti, A & E Costa: Tissue concentration of *p*-hydroxynorephedrine in rats injected with d-amphetamine: Effect of pretreatment with desipramine. *Life Sci.* 1969, 8, 653–665.

Iversen, L. L.: Accumulation of α-methyltyramine by the noradrenaline uptake process in the isolated rat heart. *J. Pharm. Pharmacol.* 1966, 18, 481–484.

Kopin, I. J., J. E. Fischer, J. M. Musacchio, W. D. Horst & V. K. Weise: False neurochemical transmitters and the mechanism of sympathetic blockade by monoamine oxidase inhibitors. *J. Pharmacol. exp. Ther.* 1965, **147**, 186–193.

Kopin, I. J.: False adrenergic transmitters. *Ann. Rev. Pharmacol.* 1968, 8, 377–393.

Lewander, T.: Effects of amphetamine on urinary and tissue catecholamines in rats after inhibition of its metabolism with desmethylimipramine. *Europ. J. Pharmacol.* 1968, **5**, 1–9.

Lewander, T.: Catecholamine turn-over studies in chronic amphetamine intoxication. In: *International symposium on amphetamines and related compounds.* Ed.: E. Costa and S. Garattini. Raven Press. New York. 1970.

Lewander, T.: On the presence of *p*-hydroxynorephedrine in the rat brain and heart in relation to changes in catecholamine levels after administration of amphetamine. *Acta pharmacol. et toxicol.* 1971, **29**, 33–48.

Maître, L. & H. Brunner: Antihypertensive und katecholamin-entleernde Wirkungen von α-Methyl-tyramin und α-Methyl-octopamin. *Arch. Pharmakol. Exptl. Path.* 1967, **257**, 40.

Randrup, A. & I. Munkvad: Biochemical, anatomical and psychological investigations of stereotyped behaviour induced by amphetamines. In: *International symposium on amphetamines and related compounds.* Ed.: E. Costa and S. Garattini. Raven Press. New York. 1970.

Thoenen, H., A. Hürliman, K. F. Gey & W. Haefely: Liberation of *p*-hydroxynorephedrine from cat spleen by sympathetic nerve stimulation after pretreatment with amphetamine. *Life Sci.* 1966, 5, 1715–1722.

Acta pharmacol. et toxicol. 1971, **29**, 33–48.

From the Psychiatric Research Center, Ulleråker Hospital,
University of Uppsala, Uppsala, Sweden

On the Presence of *p*-Hydroxynorephedrine in the Rat Brain and Heart in Relation to Changes in Catecholamine Levels after Administration of Amphetamine

By

Tommy Lewander

(Received May 28, 1970)

Abstract: The brain and heart NA in rats was maximally decreased by 28–52 % at 3–12 hours after 20 mg/kg intraperitoneally of dl-amphetamine-SO_4. Control levels of NA were not reached until 96 hours after a single injection of amphetamine, while the drug disappeared from the brain and plasma within 12 hours. Amphetamine was found to disappear from rat tissues in a poly-phasic pattern after intraperitoneal administration. The brain/plasma ratio was 9.0 ± 0.3 (mean ± S. D.). Parahydroxynorephedrine was identified in the brain and heart as a metabolite of the d-isomer of amphetamine. Parahydroxynorephedrine, the apparent $T_{1/2}$ of which was estimated to be about 22 hours, seems to be responsible for the prolonged depletion in the brain and heart NA levels caused by amphetamine. However, there was not an exact stoichiometric relation between the NA deficit and the amount of *p*-hydroxynorephedrine present. After pretreatment with desmethylimipramine, which inhibits the parahydroxylation of amphetamine, the NA in the brain and heart was decreased by amphetamine but already returned to control levels in about 12 hours. It is concluded that the persistent depletion of brain and heart NA induced by amphetamine is caused by the incorporation of *p*-hydroxynorephedrine as a false transmitter into NA neurons.

Key-words Rat – brain – catecholamines – amphetamine

In a previous study (Lewander 1968a) long-term administration of dl-amphetamine sulphate (16–48 mg/kg intraperitoneally) to rats was found to cause a progressive decrease in the tissue levels of NA* and DA. After an initial increase in urinary excretion of adrenaline and noradrenaline there was a gradual decrease in the excretion during chronic amphetamine treatment indicating tolerance to the drug. It was suggested as a tentative explana-

* Abbreviations: NA = noradrenaline, DA = dopamine, CA = catecholamine(s).

tion of these findings that amphetamine or one of its metabolites could displace the tissue stores of catecholamines and possibly serve as a false transmittor.

This suggestion was based on the finding of radioactively labelled *p*-hydroxynorephedrine in the heart but not in the brain of rats injected with d-amphetamine-^{3}H (GOLDSTEIN & ANAGNOSTE 1965). Furthermore, THOENEN *et al.* (1966) had shown that *p*-hydroxynorephedrine-^{3}H and norephedrine-^{3}H is formed from ampheamine-^{3}H in the cat spleen and that *p*-hydroxynorephedrine can be released by nerve stimulation.

In a preliminary study (LEWANDER 1970) *p*-hydroxynorephedrine was found in the rat brain after the administration of amphetamine.

The present paper deals with the elimination rate of amphetamine in the rat, the conversion of amphetamine to *p*-hydroxynorephedrine *in vivo* and the relationship between the presence of *p*-hydroxynorephedrine and the amphetamine-induced depletion of NA in the brain and heart tissues.

Material and Methods

Male Sprague-Dawley rats, 200 g body weight, were kept in individual cages and were provided with food and water *ad libitum.* The sulphate salts of dl-, d- or l-amphetamine, dissolved in sterile saline, were injected intraperitoneally in the doses given in the text. The dl-, d- and l-forms of amphetamine-7-^{14}C sulphate were obtained from CEA, Saclay, Gif-sur-Yvette, France.

Determination of amphetamine by gas liquid chromatography (GLC)

The brain, liver and lung tissues were homogenized in 0.4 M perchloric acid, 6 ml/g tissue, and centrifuged at 10,000 $\times$ g for 10 minutes. An aliquot of the supernatant was extracted with an equal volume of distilled toluene at pH 12 (5 M sodium hydroxide) for 15 minutes. The amphetamine recovered (93 $\pm$ 6 %, mean $\pm$ S. D.) in the toluene phase was reacted with trichloroacetyl chloride and the amphetamine derivative was analyzed by GLC with electron capture detection (ÄNGGÅRD *et al.* 1970). The sensitivity of the method allowed the determination of amphetamine levels of 10–20 ng/g tissue.

Determination of NA and DA.

Single brains or hearts were homogenized in 10 ml of 0.4 M perchlorid acid. After centrifugation, 10,000 $\times$ g for 10 min., the supernatant was adjusted to pH 4 with 2 M potassium hydroxide and recentrifuged. After a second adjustment of the pH to 8.5 with 1 M Tris-buffer, NA and DA were adsorbed on alumina (600 mg) columns and eluted with 0.2 M acetic acid. NA in the eluate was assayed fluorimetrically according to CHANG (1964) and DA according to CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962).

Determination of p-hydroxynorephedrine

Rats were injected intraperitoneally with 20 mg/kg dl-amphetamine-7-^{14}C sulphate, the specific activity of which was 4.5 μci per μg of the base (in total 15 μci per rat) after dilution with stable amphetamine. To the perchloric acid homogenates of the brains

and hearts 20–40 μg each of dl-amphetamine, p-hydroxyamphetamine and dl-norephedrine and p-hydroxynorephedrine were added. After centrifugation at 10,000 × g for 10 min. at +4°, the supernatants were adjusted to pH 4.5 with 2 M potassium hydroxide and recentrifuged. The samples were put on strong cation exchange resin columns (Amberlite CG 120, type II, Na^+-form, 0.4 × 5 cm). After washing the column with 5 ml redestilled water, 5 ml of 1 M sodium acetate buffer pH 6.0 containing 0.1 % EDTA and then again with 10 ml of redestilled water, the amines were eluted with 1 M hydrochloric acid in 1.3 ml fractions. The fractions containing p-hydroxynorephedrine and p hydroxyamphetamine were localized by reading each fraction at 285/335 nm (uncorrected) in an Aminco-Bowman spectrophotofluorometer, against standard solutions (1–5 μg/ml M-HCl) of the two amines. Portions of each eluate were taken for the measurement of radioactivity as described below.

During the development of the ion-exchange chromatographic method, it was found that norephedrine appeared in approximately the same fractions as p-hydroxynorephedrine (fraction 4–15, fig. 3a) and that amphetamine-^{14}C appeared immediately afterwards and partly overlapping the p-hydroxyamphetamine peak.

For determinations the portions of the eluate containing p-hydroxynorephedrine-norephedrine (fractions 4–15) and p-hydroxyamphetamine-amphetamine (fractions 15–60) respectively were pooled, evaporated and redissolved in methanol. The compounds in the first portions of the eluate were then separated by thin-layer chromatography on ChromAR® 500 sheets (5 × 16 cm, Malinckrodt), run in benzene-pyridine-acetic acid (30/1/10) or by paperchromatography according to Ellison *et al.* (1966). The amines were visualized by spraying the chromatograms with a 0.2 % ninhydrine solution in acetone containing 1 % (v/v) pyridine. Radiochromatograms of the thin-layer and paper strips were prepared by cutting them into 0.5–1 cm segments which were put into counting vials Recovery of p-hydroxynorephedrine was 92 ± 3.9 % (mean ± S. D.).

The residue of the second portion of the eluate was transferred directly into counting vials and was regarded as amphetamine-^{14}C.

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer as described previously (Lewander 1968a). Quenching was monitored by the external standard technique.

The sequential analysis of p-hydroxynorephedrine with ion exchange chromatography followed by TLC was necessary, since dl-amphetamine-7-^{14}C was found to contain varying amounts (0.5–7 %) of impurities that followed norephedrine in the chromatographic system used. In some experiments (see "Results") the ^{14}C-labelled amphetamine was purified to > 99.8 % by means of the ion-exchange chromatographic method described above.

Tissue levels of CA, amphetamine and its metabolites were calculated as the respective bases.

Comparisons of data were made by use of Student's t-test.

Results

Time-course of changes in CA levels after amphetamine.

The brain and heart NA and the brain DA were determined at intervals after the i. p. injection of 20 mg/kg dl-amphetamine-SO_4 (fig. 1). It was found that both the brain and heart NA levels were decreased by a maximum of 28 % at 3 hours in the brain, and by 52 % at 12 hours in heart tissue.

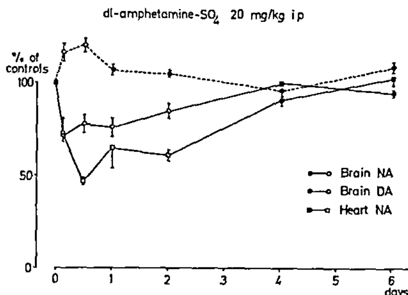


Fig. 1. The time-response relationships of the changes in brain NA, brain DA and heart NA levels after dl-amphetamine. Controls set to 100 %. Each point represents the mean (± S. E. M.) of 4–6 determinations. Open symbols indicate statistically significant ($P < 0.05$ or less) differences from the control level. Absolute values for control levels of catecholamines were: Brain NA = 0.39 ± 0.01 µg/g, brain DA = 0.79 ± 0.04 µg/g and heart NA = 0.77 ± 0.04 µg/g.

shorter time intervals the decrease was submaximal in both tissues. Control levels of NA were not reached until 96 hours after the administration of amphetamine for both the brain and heart.

There was an increase of 16–20 % in the brain DA at 3 and 12 hours, but otherwise no statistically significant changes in brain DA were observed.

Table 1.

Amphetamine concentrations (mean ± S. E. M.) in rat tissues at 0.5 hour after an intraperitoneal injection of 20 mg/kg dl-amphetamine-SO_4 and $T_{1/2}$ for the initial and late phases of disappearance of the drug from the tissues.

Tissue	Amph. conc. 0.5 hr after injection	$T_{1/2}$ in minutes	
		Initial phase 0.5–2 hrs *after inj.*	Late phase 4–12 hrs *after inj.*
Brain	19.3 ± 2.1	36	120
Plasma	2.22 ± 0.32	33	135
Liver	14.6 ± 1.2	36	175
Lung	41.6 ± 1.3	42	120

Disappearance rates of amphetamine in rat tissues.

The levels of amphetamine in the brain, plasma, liver and lung tissue were determined by GLC at different intervals after the administration of amphetamine (20 mg/kg intraperitoneally). As shown in fig. 2, the disappearance of amphetamine from both brain and plasma was multiexponential. Similar curves were obtained for liver and lung tissues. The initial and late $T_{1/2}$-values, determined graphically from semilogarithmic plots, are given in table 1 together with the initial absolute amphetamine levels.

In contrast to the prolonged changes in the brain and heart NA levels caused by the amphetamine injection (see above), neither unlabelled (GLC) nor radioactive labelled amphetamine could be detected in the brain later than 12 hours after the injection. The brain/plasma ratio: 9.0 ± 0.3 (mean ± S. D.) was fairly constant during the 12 hours after the injection of amphetamine.

Identification of p-hydroxynorephedrine as a metabolite of amphetamine in the brain and heart.

Amphetamine-^{14}C (20 mg/kg, with a radiochemical purity of $>$ 99.8 %, see "Methods") was injected 3 hours before sacrifice of the animals and the

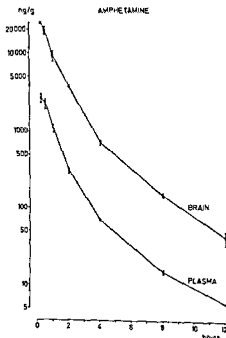


Fig. 2. Disappearance of dl-amphetamine from brain and plasma after one intraperitoneal injection of dl-amphetamine-SO_4, 20 mg/kg, at zero time. Each point represent the mean (± S. E. M.) of 3 determinations.

perchloric acid extracts of the brain and heart tissues were submitted to the sequential analysis of *metabolites of amphetamine as described in Methods.* The ion-exchange chromatography of the brain extract is shown in fig. 3a.

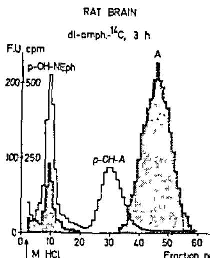


Fig. 3a. Ion-exchange chromatography of a brain extract from 2 pooled rats injected with dl-amphetamine-^{14}C, 3 hours previously Solid line: Native fluorescence (285/335 nm) in fluorescence units (F. U.)/ml of the eluate. Broken line (= shaded area): Radioactivity in cpm/fraction. Abbreviations: *p*-OH-NEph = *p*-hydroxynorephedrine, *p-OH-A* = *p*-hydroxyamphetamine, A = amphetamine.

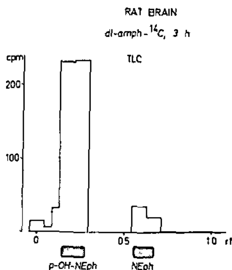


Fig. 3b. Thin-layer chromatography of fractions 6–15 from the ion-exchange chromatogram (fig. 3a). *Abbreviations: See fig. 3a,* NEph = *norephedrine.* Hatched areas indicate ninhydrin-stained spots of authentic *p*-OH-NEph and NEph added to the brain extract before the analysis.

The first radioactive peak, coinciding with authentic p-hydroxynorephedrine and norephedrine, was further analyzed by TLC (fig. 3b).

Approximately 90 % of the radioactivity in this peak co-chromatographed with authentic p-hydroxynorephedrine and 10 % with authentic norephe-

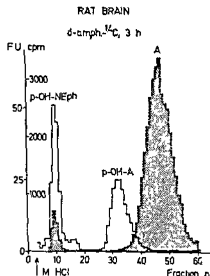


Fig. 4a Ion-exchange chromatography of a brain extract from 2 pooled rats injected 3 hours previously with d-amphetamine-^{14}C. For symbols and abbreviations see fig. 3a.

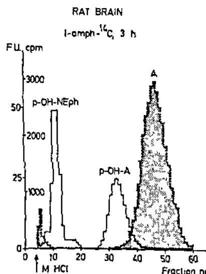


Fig. 4b. Ion-exchange chromatography of a brain extract from 2 pooled rats injected with l-amphetamine-^{14}C 3 hours previously. For symbols and abbreviations see fig. 3a.

drine. 24 hours after the injection all the radioactivity in this peak followed that of authentic *p*-hydroxynorephedrine.

TLC and paper chromatography of the fractions 25–60 of the ion-exchange chromatogram, containing the second radioactive peak, showed that practically all the radioactivity coincided with authentic amphetamine. Only negligible amounts of *p*-hydroxyamphetamine were detected. The results obtained on processing the heart extract were similar to those shown for the brain tissue extract.

In similar experiments, rats were injected with d- or l-amphetamine-7-^{14}C and sacrificed 3 hours later. It is shown in fig. 4a and 4b that *p*-hydroxynorephedrine-^{14}C was present in the brain of rats given d-amphetamine-^{14}C, while no radioactivity was detectable in the *p*-hydroxynorephedrine fractions after l-amphetamine-^{14}C. The same was true for the hearts examined.

Relationship between changes in CA levels and occurrence of p-hydroxynorephedrine in rat brain and heart.

Four groups of five rats each were injected intraperitoneally with 20 mg/kg dl-, d- or l-amphetamine-SO_4 and saline (control). The brains were analyzed for amphetamine, NA and DA. Information on the occurrence of *p*-hydroxy-

Table 2.

Brain content of amphetamine, occurrence of *p*-hydroxynorephedrine (*p*-OH-NEph) and changes in brain catecholamine levels after administration of dl-, d- and l-amphetamine *respectively.*

Treatment	Time before sacrifice	Brain			
		Amph. µg/g	*p*-OH-NEph + or −	NA %	DA %
Saline (n = 5)	3 hrs	–	–	100 ± 2.5[a]	100 ± 3.6[a]
dl-amph.-SO_4 20 mg/kg i.p. (n = 5)	3 hrs	1.17 ± 0.15	+	77 ± 5.1**	123 ± 3.6**
d-amph.-SO_4 20 mg/kg i.p. (n = 5)	3 hrs	1.40 ± 0.12	+	67 ± 5.1***	118 ± 6.0*
l-amph.-SO_4 20 mg/kg i.p. (n = 5)	3 hrs	1.27 ± 0.28	–	87 ± 2.5*	112 ± 4.8*

* Difference versus controls: $P < 0.05$

** Difference versus controls: $P < 0.01$.

*** Difference versus controls: $P < 0.001$.

[a] Control levels: brain NA = 0.39 µg/g, brain DA = 0.84 µg/g.

norephedrine were taken from the previous experiment. The results are summarized in table 2.

Three hours after the administration of amphetamine there were no differences in the brain concentrations of amphetamine between the groups. The decrease in the brain NA was most marked after d-amphetamine and less marked after dl-amphetamine. Even l-amphetamine caused a significant decrease in brain NA though p-hydroxynorephedrine was not found in these brains. It was noted that both d-, l- and racemic amphetamine caused a small but significant increase in brain DA in agreement with fig. 1.

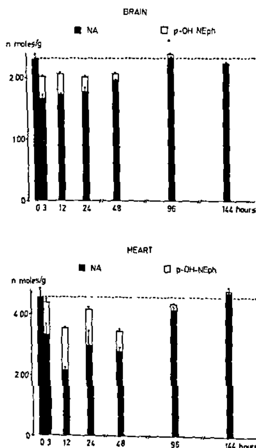


Fig. 5. Remaining stores (mean ± S. E. M.) of NA-black columns in rat brain (above) and heart (below) tissues at different time points after administration of dl-amphetamine (20 mg/kg intraperitoneally). White columns, on top of the black columns, represent the amounts of p-hydroxynorephedrine (p-OH-NEph) present in the tissues, each value representing the mean ± S E. M. of 3–4 determinations. Broken horisontal lines indicate control levels of NA.

Table 3.

Effect of reserpine on the mean (± S. E. M.) levels of *p*-hydroxynorephedrine (*p*-OH-NEph) and amphetamine (Amph.) in rat brain and heart tissues. Reserpine, 5 mg/kg intraperitoneally, was given 4 hours before amphetamine-^{14}C, 20 mg/kg intraperitoneally, and the animals were sacrificed at 2 hours after the amphetamine injection.

Tissue	Treatment	*p*-OH-NEph ng/g	Amph. µg/g
Brain	Saline + dl-amph. (4)	56 ± 1	2.88 ± 0.21
	Reserpine + dl-amph. (3)	13 ± 0.1*	2.28 ± 0.23
Heart	Saline + dl-amph. (3)	77 ± 10	1.07 ± 0.23
	Reserpine + dl-amph. (3)	24 ± 4*	1.11 ± 0.06

* Difference: $P < 0.001$.
In brackets: number of observations.

Time-response relationships between NA-depletion and amounts of p-hydroxynorephedrine in the brain and heart after dl-amphetamine.

The amounts of *p*-hydroxynorephedrine were determined in the brains and hearts of rats after the administration of dl-amphetamine-^{14}C, 20 mg/kg intraperitoneally, and compared with the magnitude and duration of the NA-depletion caused by the drug in another group of rats of the same age and body weight (cf. fig. 1). It is shown in fig. 5 that *p*-hydroxynorephedrine

Table 4.

Effects of DMI on the mean (± S. E. M.) levels of *p*-hydroxynorephedrine (*p*-OH-NEph) and amphetamine (Amph.) in rat brain and heart tissues. DMI, 10 mg/kg intraperitoneally, was given 1 hour before amphetamine-^{14}C, 20 mg/kg intraperitoneally, and the animals were sacrificed 3 hours after the amphetamine injection.

Tissue	Treatment	*p*-OH-NEph ng/g	Amph. µg/g
Brain	Saline + dl-amph (4)	59 ± 5	0.51 ± 0.07
	DMI + dl-amph. (4)	8 ± 0.1*	10.63 ± 0.53***
Heart	Saline + dl-amph (3)	180 ± 30	0.28 ± 0.02
	DMI + dl-amph. (4)	8 ± 0.1*	6.08 ± 0.09***

* Difference: $P < 0.001$.
In brackets: number of determinations.

was present in both the brain and heart tissues as long as the NA levels were depressed. However, the NA-deficit was not completely covered by the quantities of p-hydroxynorephedrine present.

Concentrations of p-hydroxynorephedrine and amphetamine in the brain and heart of reserpine treated rats.

The experiment is described in the legend to table 3. In the brains and hearts of the reserpine treated animals the amounts of p-hydroxynorephedrine were only 23 % and 31 % respectively of the control values. The concentrations of amphetamine in the brain and heart did not differ between the reserpine treated and control rats.

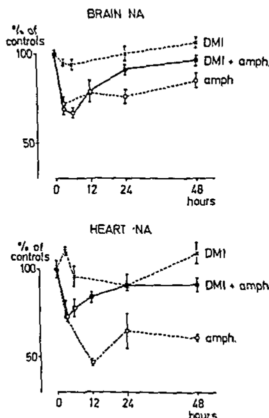


Fig. 6. The effect of DMI pretreatment on the time-response relationships of the NA depletion in rat brain and heart tissues caused by dl-amphetamine. The NA levels are expressed as percentages (mean ± S. E. M., n = 4–6) of control levels (brain NA = 0.33 ± 0.01 µg/g, brain DA = 0.85 ± 0.03 µg/g and heart NA = 0.86 ± 0.013 µg/g). Open circles indicate statistically significant (P > 0.01 or less) differences from the control values.

Concentrations of p-hydroxynorephedrine and amphetamine in the brain and heart of DMI treated rats.

The experimental design and the results are given in table 4. It is evident that after DMI pretreatment there is only a negligible amount of *p*-hydroxynorephedrine present in the brain and the heart. In contrast, the concentrations of amphetamine in the 2 tissues were increased by a factor of 20–22.

Time-course of the decrease in the brain and heart NA caused by amphetamine after DMI pretreatment.

In fig. 6 the changes in the NA levels in the brain and heart are plotted against time after administration of DMI or DMI + amphetamine. Data on the changes in tissue NA levels induced by amphetamine alone (from fig. 1) are inserted for comparison. After DMI + amphetamine, the brain NA is decreased to the same level as after amphetamine alone but returns to control values within 24 hours, while the duration of the NA depletion after amphetamine still persists. For heart NA the results were similar. DMI alone did not produce any change in the brain or heart NA levels.

Discussion

The original observation by McLean & McCartney (1961) that brain and heart NA is decreased by amphetamine in the rat has been confirmed by several investigators (Moore & Larivière 1963; Baird & Lewis 1964; Gunne & Lewander 1967; Lewander 1968a and b).

A close examination of the temporal relationships between the changes in CA levels and the concentrations of amphetamine in the tissues was one of the primary objects of the present investigations. The results showed that amphetamine disappeared from the brain and plasma within 12 hours while the brain and heart NA was decreased for about 72 hours.

The disappearence of amphetamine from the plasma and tissues was found to be polyphasic. The same observation was reported by Maickel *et al.* (1969) using various doses of d-amphetamine. No explanation for these results can be given at present. The present data on the distribution of amphetamine in the rat and the brain/plasma ratio are in good agreement with earlier reports (Maickel *et al.* 1966; Maickel *et al.* 1969).

The preliminary finding of *p*-hydroxynorephedrine as a metabolite of amphetamine in the brain (Lewander 1970) has been confirmed. Recently, Costa & Groppetti (1970), Groppetti & Costa (1969a and b) and Brodie *et al.* (1970) independently reported the presence of *p*-hydroxynorephedrine in the rat brain after administration of d-amphetamine-^{3}H. Parahydroxynorephedrine in the brain most probably originates from *p*-hydroxyamphet-

amine, which is formed from amphetamine in the rat liver (AXELROD 1954). In a previous communication (LEWANDER 1971) it was reported that peripherally administered p-hydroxyamphetamine-^{3}H is recovered in the rat brain together with p-hydroxynorephedrine-^{3}H. The failure to detect p-hydroxynorephedrine in the brain in the study of GOLDSTEIN & ANAGNOSTE (1965) may be due to the low dose of amphetamine used in their experiments.

GOLDSTEIN & ANAGNOSTE (1965) have shown that p-hydroxynorephedrine is formed in the rat heart after the administration of d-amphetamine but not after l-amphetamine. According to the present experiments this is also true for the rat brain.

THOENEN *et al.* (1966) reported the presence of norephedrine, as well as of p-hydroxynorephedrine, in the cat spleen after administration of amphetamine. With the use of highly purified amphetamine-^{14}C (see "Methods") only negligible amounts (< 10 ng/g brain) of norephedrine-^{14}C was found in the rat brain. This amount however, is at the limit of detection with the present methods. The failure to detect significant amounts of norephedrine in rat tissues is in agreement with previous reports (GOLDSTEIN & ANAGNOSTE 1965; BRODIE *et al.* 1970).

The prolonged depletion of brain and heart NA after amphetamine was related to the presence of p-hydroxynorephedrine in these tissues (fig. 5). However, there was not an exact stoichiometrical relationship between the missing NA and the tissue levels of p-hydroxynorephedrine. Most of the p-hydroxynorephedrine, like NA, however, is bound by a reserpine sensitive mechanism (BRODIE *et al.* 1970; present study). This is in contrast to the observation that the NA-deficit in rat brain and heart after the administration of p-hydroxyamphetamine is equimolar with the amounts of p-hydroxynorephedrine present (LEWANDER 1971). The finding that l-amphetamine causes a release of brain NA without being converted to p-hydroxynorephedrine (fig. 4b, table 2) partly explains the lack in stoichiometry in the present experiment with dl-amphetamine. After d-amphetamine-^{3}H (GROPPETTI & COSTA 1969b) a perfect stoichiometry was present in the brain but not in the heart of rats, which indicate that displacement of NA by p-hydroxynorephedrine is only one mechanism by which amphetamine decreases tissue NA levels.

This point is further elaborated in the experiments with DMI. Pretreatment of rats with DMI causes an almost complete inhibition of the p-hydroxylation of amphetamine (CONSOLO *et al.* 1967; LEWANDER 1968b and 1969) and is accompanied by increased tissue levels of amphetamine (SULSER *et al.* 1966; VALZELLI *et al.* 1967). In addition, DMI inhibits the uptake of p-hydroxyamphetamine into heart and brain neurons (IVERSEN 1966; LEWANDER 1968b). These effects of DMI explain the low levels of p-hydroxynorephedrine and the 20-fold increase in amphetamine in the brains and

hearts of rats given DMI + amphetamine (table 4), which is in accordance with the findings of GROPPETTI & COSTA (1969b).

The results presented in fig. 5 show that even if the formation of *p*-hydroxynorephedrine from amphetamine is blocked by DMI, there is a decrease in both heart and brain NA. The duration of the amphetamine-induced NA depletion in both brain and heart was, however, shortened. These findings lend support to the conclusion (LEWANDER 1968b) that amphetamine itself is capable of depleting tissue NA levels and the importance of its metabolite *p*-hydroxynorephedrine for the persistent NA depletion is emphasized. It is known that amphetamine, in addition to its releasing effect on extragranular NA (CARLSSON *et al.* 1965; CARLSSON *et al.* 1966) accelerates the release of granular bound NA (CARLSSON *et al.* 1965; EULER & LISHAJKO 1968). Amphetamine has, however, a poor affinity for NA binding sites in the mouse heart (OBIANWU *et al.* 1968).

DMI does not seem to block the depletion of brain and heart NA caused by amphetamine (LEWANDER 1968b and the present study). The opposite observations were made for rat hearts by BRODIE *et al.* (1968) and GROPPETTI & COSTA (1969b). Differences in dosage and time interval after amphetamine (BRODIE *et al.* 1968: 20–40 minutes and GROPPETTI & COSTA 1968b: 9 hours) may explain these apparently contradictory results. The uptake of amphetamine into brain or heart slices *in vitro* is not inhibited by DMI (ROSS & RENYI 1966; ROSS *et al.* 1968).

In agreement with earlier studies (cf. GLOWINSKI & BALDESSARINI 1967; LEWANDER 1968a and b) there was an increase in the brain DA, which in the present study was found to be of approximately the same duration as the presence of amphetamine in the brain. Racemic as well as the 2 stereoisomers of amphetamine in equal doses induced a significant increase in brain DA (table 2). In contrast very high doses of d-amphetamine (30 mg/kg intraperitoneally), dl-amphetamine given to DMI pretreated rats (see below) or chronic amphetamine administration is followed by a decrease in the brain DA (MOORE & LARIVIÈRE 1963; LEWANDER 1968a and b). Possible explanations for these different effects of amphetamine on brain DA have been discussed previously (LEWANDER 1968a and b).

In summary it appears from the present results that the displacement of brain and heart NA caused by *p*-hydroxynorephedrine, formed from amphetamine, may contribute to the marked reduction in tissue NA levels and the decreased urinary CA excretion seen after chronic amphetamine treatment (LEWANDER 1968a).

Acknowledgements

The author is indebted to Mrs. Inger Jansson and Miss Sonja Ahlén for skilful technical assistance. The study was supported by the Swedish Medical

Research Council (project No. B70-40X-1017-06B and B70-27P-2627-02) and the Tri-Centennial Fund of the Bank of Sweden (project No. 150).

REFERENCES

Änggård, E., L.-M. Gunne & F. Niklasson: Gas chromatographic determination of amphetamine in blood, tissue and urine. *Scand. J. Clin. Lab. Invest.* 1970, in press.

Axelrod, J.: Studies on sympathomimetic amines. II. The biotransformation and physiological disposition of d-amphetamine, d-, p-hydroxyamphetamine and d-methamphetamine. *J. Pharmacol. exp. Ther.* 1954, **214**, 315–326.

Baird, J. R. C. & J. J. Lewis: The effects of cocaine, amphetamine, and some amphetamine-like compounds on the *in vivo* levels of noradrenaline and dopamine in the rat brain. *Biochem. Pharmacol.* 1964, **13**, 1475–1482.

Brodie, B. B., E. Costa, A. Groppetti & C. Matsumoto: Interaction between desipramine, tyramine, and amphetamine at adrenergic neurones. *Brit. J. Pharmacol.* 1968, **34**, 648–658.

Brodie, B. B., A. K. Cho & G. G. Gessa: Possible role of p-hydroxynorephedrine in the depletion of norepinephrine induced by d-amphetamine and in tolerance to this drug. In: *International symposium on amphetamine and related compounds.* Milano, March 1969. Ed.: E. Costa & S. Garattini. Raven Press, New York, 1970.

Carlsson, A. & B. Waldeck: A fluorimetric method for the determination of dopamine (3-hydroxytyramine). *Acta physiol. scand.* 1958, **44**, 293–298.

Carlsson, A. & M. Lindqvist: *In vivo* decarboxylation of α-methyldopa and α-methyltyrosine. *Acta physiol. scand.* 1962, **54**, 87–94.

Carlsson, A., M. Lindqvist, A. Dahlström, K. Fuxe & D. Masuoka: Effects of the amphetamine group on intraneuronal brain amines *in vivo* and *in vitro*. *J. Pharm. Pharmacol.* 1965, **17**, 521–523.

Carlsson, A., K. Fuxe, B. Hamberger & M. Lindqvist: Biochemical and histochemical studies on the effects of imipramine-like drugs and (+)-amphetamine on central and peripheral catecholamine neurons. *Acta physiol. scand.* 1966, **67**, 481–497.

Chang, C. C.: A sensitive method for spectrophotofluorimetric assay of catecholamines. *Int J. Neuropharmacol* 1964, **3**, 643–649.

Consolo, S., E. Dolfini, S. Garattini & L. Valzelli: Desipramine and amphetamine metabolism, *J. Pharm. Pharmacol.* 1967, **19**, 253–256.

Costa, E & A. Groppetti: Biosynthesis and storage of catecholamines in tissues of rats injected with various doses of *d*-amphetamine. In: *International symposium on amphetamines and related compounds.* Milano, March 1969. Ed.: E. Costa & S. Garattini. Raven Press, New York, 1970.

Ellison, T., L. Gutzait & E. J. van Loon: The comparative metabolism of d-amphetamine-^{14}C in the rat, dog and monkey. *J. Pharmacol. exp. Ther.* 1966, **152**, 383–387.

Euler, U. S. v. & F. Lishajko: Effect of directly and indirectly acting sympathomimetic amines on adrenergic transmitter granules. *Acta physiol. scand.* 1968, **73**, 78–92.

Glowinski, J. & R. J. Baldessarini: Metabolism of norepinephrine in the central nervous system. *Pharmacol. Rev.* 1967. **18**, 1201–1238.

Goldstein, M. & B. Anagnoste: The conversion *in vivo* of D-amphetamine to (+)-p-hydroxynorephedrine. *Biochim. Biophys. Acta.* 1965, **107**, 166–168.

Groppetti, A. & E. Costa: d-Amphetamine (A): Metabolites and depletion of brain and heart norepinephrine (NE) in guinea pig and rat. *Fed. Proc.* 1969a, **28**, 795.

Groppetti, A. & E. Costa: Tissue concentration of *p*-hydroxynorephedrine in rats injected with d-amphetamine: Effect of pretreatment with desipramine. *Life Sci.* 1969b, 8, 653–665.

Gunne, L.-M. & T. Lewander: Long-term effects of some dependence-producing drugs on the brain monoamines. In: *Molecular basis of some aspects of mental activity.* Academic Press, London and New York, 1967, 2, 75–81.

Iversen, L. L.: Accumulation of α-methyltyramine by the noradrenaline uptake process in the isolated rat heart. *J. Pharm. Pharmacol.* 1966, 18, 481–484.

Lewander, T.: Urinary excretion and tissue levels of catecholamines during chronic amphetamine intoxication. *Psychopharmacologia* 1968a, 13, 394–407.

Lewander, T.: Effects of amphetamine on urinary and tissue catecholamines in rats after inhibition of its metabolism with desmethylimipramine. *Europ. J. Pharmacol.* 1968b, 5, 1–9.

Lewander, T.: Influence of various psychoactive drugs on the *in vivo* metabolism of d-amphetamine in the rat. *Europ. J. Pharmacol.* 1969, 6, 38–44.

Lewander, T.: Catecholamine turn-over studies in chronic amphetamine intoxication. In: *International symposium on amphetamines and related compounds*, Milano, March, 1969. Ed.: E. Costa & S. Garattini. Raven Press, New York, 1970.

Lewander, T.: Displacement of brain and heart noradrenaline by *p*-hydroxynorephedrine after administration of *p*-hydroxyamphetamine. *Acta pharmacol. et toxicol.* 1971, 29, 20–32.

Maickel, R. P., R. H. Cox Jr., D. S. Segal & F. P. Miller: Studies on psychoactive drugs. I. Physiological disposition and time course of behavioural effects of d-amphetamine in rats. *Fed. Proc* 1966, 25, 385.

Maickel, R. P., R. H. Cox Jr., F. P. Miller, D. S. Segal & R. W. Russel: Correlation of brain levels of drugs with behavioural effects. *J. Pharmacol. exp. Ther.* 1969, 165, 216–224.

McLean, J. R. & M. McCartney: Effect of d-amphetamine on rat brain noradrenaline and serotonin. *Proc. Soc. exp. Biol.* (N.Y.). 1961, 107, 77–79.

Moore, K. E. & E. W. Larivière: Effects of d-amphetamine and restraint on the content of norepinephrine and dopamine in rat brain. *Biochem. Pharmacol* 1963, 12, 1283–1288.

Obianwu, H. O., R. Stitzel & P. Lundborg: Subcellular distribution of ^{3}H-amphetamine and ^{3}H-guanethidine and their interaction with adrenergic neuron. *J. Pharm. Pharmacol.* 1968, 20, 585–594.

Ross, S. B & A. L. Renyi: Uptake of some tritiated sympathomimetic amines by mouse brain cortex slices *in vitro*. *Acta pharmacol. et toxicol.* 1966, 24, 297–309.

Ross, S. B., A. L. Renyi & B. Brunfelter: Cocainesensitive uptake of sympathomimetic amines in nerve tissue. *J. Pharm. Pharmacol.* 1968, 20, 283–288.

Sulser, F., M. L. Owens & J. V. Dingell: On the mechanism of amphetamine potentiation by desipramine (DMI). *Life Sci.* 1966, 5, 2005–2010.

Thoenen, H., A. Hürliman, K. F. Gey & W. Haefely: Liberation of *p*-hydroxynorephedrine from cat spleen by sympathetic nerve stimulation after pretreatment with amphetamine. *Life Sci.* 1966, 5, 1715–1722.

Valzelli, L., S. Consolo & C. Morpurgo: Influence of imipramine-like drugs on the metabolism of amphetamine. In: *Antidepressant drugs*. Ed.: S. Garattini & M. N. G. Dukes. Excerpta Medica Foundation, Amsterdam, New York, London, Milan, Tokyo, Buenos Aires. 1967, p. 61–69.

Acta pharmacol. et toxicol. 1971, **29**, 49–59.

From the Institute of Physiology, University of Oslo, Oslo, Norway

The Effect of Thioridazine and Promazine in Reducing the Potassium Loss from Isolated Perfused Rat Hearts

By

Knud Landmark

(Received June 10, 1970)

Abstract: Electrically stimulated isolated rat hearts were perfused with a Ringer solution containing a low concentration of K^+. At the end of a control period of 20 min. the cumulative K^+ loss from the hearts was approximately 30 meq./kg dry weight. This value was used in each experiment as reference (100 per cent) for the total loss found after a subsequent additional test period of 30 min. The addition of promazine and thioridazine to the perfusate at the end of the control period reduced the further K^+ loss. The values found in the different groups at the end of the test period were: Control group (10 hearts): 154 ± 11.6 per cent; with promazine 10^{-5} M added (8 hearts): 103 ± 5.6 per cent; with promazine 2.5×10^{-5} M added (10 hearts): 74 ± 10.5 per cent and with thioridazine 10^{-5} M added (8 hearts): 110 ± 5.8 per cent. In another type of experiments, ouabain 1.5×10^{-6} M was added at the end of the control period. This drug caused an increased K^+ loss (181 ± 14.2 per cent) and ventricular fibrillation in 6 out of 8 hearts. Promazine 2.5×10^{-5} M added before ouabain, prevented the development of fibrillation in another group of 7 hearts, and also markedly reduced the K^+ loss. It is concluded that phenothiazines reduce the K^+ permeability of the heart muscle membranes.

Key-words: Potassium loss – phenothiazine derivatives – ouabain – ventricular fibrillation.

Changes in the electrocardiogram (ECG) have often been observed in patients during treatment with drugs of the phenothiazine group. A review of these alterations has previously been given (LANDMARK *et al.* 1969; LANGSLET 1969).

ECG changes similar to those reported above have also been found in isolated, perfused rat hearts after the addition of phenothiazines to the

* Research Fellow, The Norwegian Council on Cardiovascular Diseases. Present Address: Institute of Pharmacology, University of Oslo.

perfusate (LANDMARK *et al.* 1969; LANGSLET 1969). The cardiac effect of these drugs thus appears to be independent of intact nervous and hormonal influences. It has been suggested by these authors that the drugs may have a direct action on the heart and that they exert their cardiac action by reducing the ionic permeability of the cell membrane. The aim of the present work has been to investigate the effect of phenothiazines on the net loss of K^+ from cardiac cells in retrogradely perfused rat hearts. The results indicate that these drugs reduce the K^+ permeability of the cardiac cell membrane.

Methods

Female albino Wistar rats (about 300 g) were used. The hearts were excised under ether anaesthesia and the aorta was cannulated as described by ØYE (1965). The hearts were perfused for 5 minutes with a cold perfusate in order to wash out blood, and then transferred to a perfusion unit (fig. 1). The perfusion medium contained the following ions (meq./l): sodium 144.4; potassium 0.8, calcium 1.7; magnesium 2.3; chloride 119.5; phosphate ($H_2PO_4^-$) 2.4; bicarbonate 25.0, and sulphate 2.3. The perfusate also contained 90 mg/100 ml of glucose, and it was gassed with 95 % O_2 and 5 % CO_2. The temperature was kept at 32°, and the pressure above the aortic valves was maintained at 75 cm of water. The K^+ content was kept low as this made it easier to detect minor differences in the concentrations of this ion in the perfusion fluid. As a consequence of the lowered K^+ concentration, it was also necessary to reduce the Ca^{++} content in order to prevent fibrillations of the hearts (GRUMBACH *et al.* 1954; ARMITAGE *et al.* 1957; BURN 1958).

The hearts were electrically stimulated with square wave pulses delivered from a laboratory stimulator (American lab. electronics, model no. 194 A). One platinum electrode was attached to the left atrium, the other was placed directly on the wall of the ventricle. Square wave pulses of 10–15 v, 0.5 m/sec. duration and a stimulation frequency of 180/min. were used. When the hearts had been perfused for 3 minutes, the cannula from reservoir R_1 (fig. 1) was clamped, and the hearts were perfused from reservoir R_2 (the pressure was above the aortic valves, the temperature and the composition of the perfusate were as before). At the same time the cannula draining the perfusion chamber was clamped, and continuous recirculation of the perfusate was started. The recirculating perfusate volume was 58 ml. Samples for potassium assay were withdrawn 10, 20, 30, 40 and 50 min. respectively after the start of the recirculation.

Before the addition of the drugs there was a control period of 20 min. These drugs were added at the top of the oxygenator in amounts giving final concentrations of 10^{-5} M, 2.5×10^{-5} M and 5×10^{-5} M of promazine and 10^{-5} M and 2.5×10^{-5} M of thioridazine. These concentrations were chosen because they had been found to produce ECG-changes in isolated rat hearts (LANDMARK *et al.* 1969). The pH of the perfusate was 7.4; the addition of the phenothiazine derivatives did not change this value.

When the perfusion was finished, the hearts were blotted on filter paper and weighed. They were then cut into pieces and dried at 60° for 24 hrs in order to estimate their dry weight.

The potassium content of the perfusate was determined with an Eppendorf flame photometer, and the K^+ loss from the hearts was calculated in meq./kg dry weight.

Results

1. *Effects of phenothiazines on net K^+ loss from isolated perfused electrically driven hearts.*

The excised hearts resumed spontaneous contractions during the first 1–2 min. in the perfusator. Electrical stimulation was started after 2 min., and the hearts were then driven at a rate of 180 beats/min. The addition of 5×10^{-5} M promazine caused atrioventricular dissociation (1 experiment),

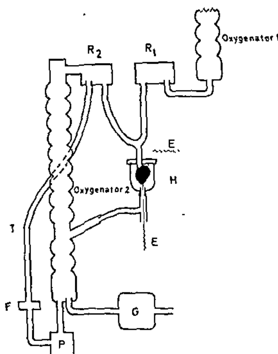


Fig. 1. Perfusion arrangement. The isolated rat heart, mounted in the perfusion chamber H, is supplied for the first 3 minutes with a Ringer solution delivered from the oxygenator 1 and the reservoir R_1. The perfusion fluid is drained through the aorta and the vascular bed, and then out at the bottom of the chamber. When the recirculation of the Ringer solution is started, both the cannula from reservoir R_1 and the cannula draining the perfusion chamber are clamped.
The Ringer solution in the oxygenator 2 is continously circulated by means of a pump (P) through a Millipore filter (F) and the tube T into the reservoir R_2, from which excess fluid flows back along the inner surface of the oxygenator 2. The gas is led in at the bottom of the oxygenator from the moistening chamber G. The perfusion fluid is drained throught the heart and back to the oxygenator 2 from reservoir R_2.
The perfusion circuit is surrounded by a water jacket through which water from a thermostatically-controlled water bath (32°) is continously circulated. The stimulating electrodes E are attached to the left atrium and the ventricle wall.

while 2.5 × 10^{-5} M thioridazine reduced the coronary flow, and ventricular fibrillation ensued (3 experiments). No further experiments with these concentrations of the two phenothiazines were therefore performed. The addition of 10^{-5} M and 2.5 × 10^{-5} M of promazine and 10^{-5} M of thioridazine did not interfere with the electrically induced rhythm or change the coronary flow, and the effects of such additions on K^+ loss from the hearts were therefore studied.

With the low concentration of K^+ in the perfusate, the hearts showed a continuous loss of this ion. The K^+ loss was highest during the first 10 min. of the perfusion. The loss then gradually decreased after which it increased a little towards the end of the experiments (fig. 2). Thioridazine markedly reduced the K^+ loss. When promazine was added, the K^+ concentration of the perfusate was reduced, thus indicating a net gain of K^+ into the hearts. This was most marked with the highest concentration of promazine used.

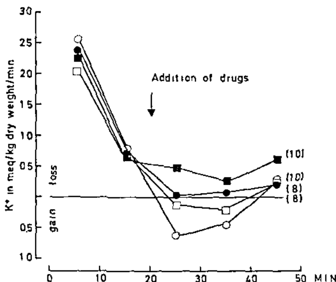


Fig. 2. The effect of promazine and thioridazine on the K^+ loss or gain from retrogradely perfused rat hearts.

Samples for K^+ assay were taken at 10 min. intervals (i. e. 10, 20, 30, 40 and 50 min. respectively after the start of the recirculation). The values given along the ordinate show the mean rate of K^+ loss or gain in meq./kg dry weight/min. in each 10 min. perfusion period. The drugs were added when the hearts had been perfused with recirculating Ringer solution for 20 min (control period). (In brackets number of experiments)

Symbols:

■——■ control hearts;

●——● 10^{-5} M *thioridazine in perfusate,*

□——□ 10^{-5} M promazine and

○——○ 2.5 × 10^{-5} *M promazine in perfusate.*

Table 1.

K⁺ loss during control period (0–20 min.).	
Control group	33.7 ± 3.8 (10)
Promazine group (10^{-5} M)	27.7 ± 2.9 (8)
Promazine group (2.5×10^{-5} M)	29.5 ± 1.6 (10)
Thioridazine group (10^{-5} M)	30.2 ± 2.3 (8)

The total (cumulative) loss of myocardial K^+ (mean value ± S. E. M.) in meq./kg dry weight at the end of the control period. (In brackets number of experiments.)

The total (cumulative) K^+ loss at the end of the first 20 min. perfusion period (control period) varied from 33.7–27.7 meq./kg dry weight in the four experimental groups (table 1).

With the value for cumulative K^+ loss at the end of the control period set at 100 per cent (in each experiment) the effect of the phenothiazines on any further K^+ loss in the subsequent test perfusion period can be demonstrated as seen in fig. 3.

2. *Protection against ouabain-induced fibrillation.*

In another series of experiments ouabain (g-strophantinum NFN), giving a final concentration of 1.5×10^{-6} M, was added 20½ min. after recirculation of the perfusate had been started. The drug caused an increased K^+ loss, and fibrillation developed for short or long periods in six out of eight hearts. Promazine 2.5×10^{-5} M added before ouabain, prevented the development of fibrillation in a group of seven hearts. Such a pre-addition also markedly reduced the K^+ loss.

Table 2 gives the total (cumulative) K^+ loss at the end of the control period in the two groups of hearts in this series of experiments.

Fig. 4 illustrates the K^+ loss found in the test perfusion periods in the presence of either ouabain alone or of ouabain and promazine together. The cumulative K^+ loss in each experiment at the end of a preceding 20 min. control period has been called 100 per cent and used as reference.

Discussion

The results presented show that 10^{-5} M and 2.5×10^{-5} M of promazine and 10^{-5} M of thioridazine were capable of reducing the K^+ loss from isolated rat hearts perfused under standardized conditions and at low perfusate levels of K^+. Several workers have discussed the possibility that chlorpromazine and

other phenothiazines produce some of their effects by acting on cell membranes (review: GUTH & SPIRTES 1964), and thereby influencing ionic permeability. FREEMAN & SPIRTES (1963) and SEEMAN & WEINSTEIN (1966) have studied the effects of phenothiazines on erythrocytes. They found that in low concentrations these drugs reduced the efflux of K^+ and the influx of Na^+, thus protecting against hypotonic haemolysis. The drugs seemed to have a general stabilizing effect on the erythrocyte membrane.

The total (cumulative) K^+ loss from isolated perfused rat hearts is the difference between the total K^+ efflux and the total active K^+ uptake. It has been shown that K^+ efflux at low $[K^+]_o$ is decreased in spite of an increased concentration gradient of outward K^+ flow (CARMELIET 1961; LANGER &

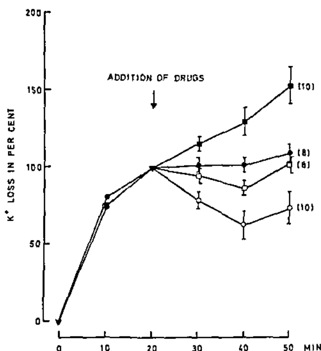


Fig. 3. The effect of promazine and thioridazine on the K^+ loss from retrogradely perfused rat hearts. The total (cumulative) loss at the end of the first 20 min. perfusion period (control period) in each experiment has been set at 100 per cent and used as reference. The values for total (cumulative) loss found in the subsequent perfusion period (mean value ± S. E. M.) have been related to this reference, so that values below 100 per cent indicate a net gain of K^+ into the hearts. (In brackets number of experiments).

Symbols:

■———■ control hearts;
●———● 10^{-5} M thioridazine in perfusate;
□———□ 10^{-5} M promazine and
○———○ 2.5×10^{-5} M promazine in perfusate.

Table 2.

K^+ loss during control period (0–20 min.).	
Hearts to which ouabain had been added	45.6 ± 2.2 (8)
Hearts to which ouabain and promazine had been added	43.7 ± 2.7 (7)

The total (cumulative) loss of myocardial K^+ (mean value ± S. E. M.) in meq./kg dry weight at the end of the control period. (In brackets number of experiments.)

BRADY 1966). This has been interpreted as a true reduction in P_K at low $[K^+]_o$ (LANGER 1968). In our experiments low concentrations of both $[K^+]_o$ and $[Ca^{++}]_o$ were perfused. If K^+ efflux was reduced under these conditions, the results indicated that K^+ influx was reduced to a greater extent since a cumulative loss of K^+ was found. This assumption is in agreement with the findings of KLEIN *et al.* (1960) who demonstrated a large reduction of K^+ influx in isolated rabbit atria at low $[K^+]_o$ while the decrease in K^+ efflux was less pronounced.

The question arises whether the effects of the phenothiazines on the total K^+ loss may be due to a decreased K^+ efflux or to an increase in the active ion transport mechanism. FREEMAN & SPIRTES (1963) have concluded that although the effect of chlorpromazine on ionic movements in erythrocytes appears to be a passive phenomenon, activation or protection of the existing active transport mechanism in the erythrocyte membrane would also explain their results. However, the effect of promazine and thioridazine on Na^+/K^+-activated adenosinetriphosphatase (ATPase) prepared from rat hearts, does not support this last possibility (LANDMARK & ØYE 1971). Concentrations of either drug comparable to those used in the present studies, caused a slight or moderate inhibition of the enzyme, while higher concentrations had a pronounced inhibitory effect. No activation or stimulation of Na^+/K^+-activated ATPase could be observed in these *in vitro* experiments. CHRISTENSEN *et al.* (1958) have found that chlorpromazine inhibited the entrance of both radioactive Na^+ and K^+ into brain tissue. Their results thus indicate that no activation of the active transport mechanism occurs when chlorpromazine is added.

Several authors (DAVIS & BRODY 1966; HUSTON & BELL 1966; LEESTMA & KOENIG 1968) have suggested that the phenothiazine derivatives produce some of their cardiac effects and complications by inhibiting the active cationic transport system. If this were the mode of action of these drugs, then an increased K^+ loss would be expected. In the present experiments, however, it was found that the K^+ loss from the rat hearts was reduced after the addition of the phenothiazines, and that if the promazine concentrations of the perfusate were increased, there could even be a net cellular gain of K^+.

It has been shown that several phenothiazine derivatives exert a negative inotropic influence on isolated heart preparations (MELVILLE & DRAPEAU 1958; HAMACHER & HILDEBRANDT 1964; LANDMARK *et al.* 1969). It seems possible that a reduction in the strength of the contractions may be the cause of the reduced K^+ loss as has been discussed by PAK *et al.* (1966). However, LANGSLET (1970) has also described a reduced K^+ loss in the presence of low concentrations of chlorpromazine (1.3×10^{-5}–1.9×10^{-4} M) from muscle fibres of asystolic rat hearts perfused at 16°. GRUPP *et al.* (1967) have found that alterations related to the excitation cycle rather than consequences of the contraction are the major determinants of the K^+ flux in the ventricle fibers. This conclusion is supported by the results of CORABOUEF *et al.* (1969). They believe that not all of the observed K^{42} efflux in right ventricles from the rat could be explained as a result of the contraction alone.

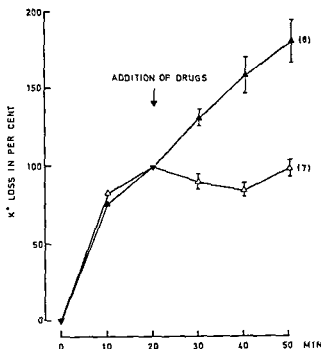


Fig. 4. The protective action of promazine on ouabain-induced K^+ loss from retrogradely perfused rat hearts. The total (cumulative) loss at the end of the first 20 min. perfusion period (control period) in each experiment has been set at 100 per cent and used as reference. The values found in the subsequent perfusion periods (mean value ± S. E. M.) have been related to this reference. Promazine was added at the end of the control period, ouabain was added 30 sec. later.

Symbols:
▲———▲ 1.5×10^{-6} M ouabain in perfusate and
△———△ 1.5×10^{-6} M ouabain and 2.5×10^{-5} M promazine.

High doses of ouabain produce ventricular arrhythmias and fibrillation *in vivo* (SEKIYA & VAUGHAN WILLIAMS 1963; DOHADWALLA *et al.* 1969). MELVILLE (1958) found that repeated doses (0.5–1.0 mg/kg) of chlorpromazine did not protect dogs against the cardiac arrhythmias which occur after prolonged infusions of ouabain. SHARMA & ARORA (1961) on the other hand, have observed that several phenothiazine derivatives (12.0–27.0 mg/kg) reverse the ouabain-induced ventricular arrhythmias in unanaesthetized dogs. The antiarrhythmic activity of phenothiazines in experimental cardiac arrhythmias has also been demonstrated by others (COURVOISIER *et al.* 1953, MADAN & PENDSE 1963; SINGH & SHARMA 1969). High concentrations of cardiac glycosides, including ouabain, depress the Na^+/K^+ activated ATPase at the cell membrane. As a result they inhibit active transmembrane cationic transport, delay the reflux of sodium and lower the intracellular potassium concentration (SCHATZMANN 1953; GLYNN 1964; WEATHERALL 1966). According to KLEIN & HOLLAND (1958) the occurrence of cardiac irregularities and fibrillation may be related to an increased K^+ efflux.

The increased K^+ loss induced by ouabain in our experiments was probably due to an inhibition of the active cationic transport. The addition of 2.5×10^{-5} M of promazine protected against ouabain-induced arrhythmias and also had a pronounced inhibitory effect on the K^+ loss from the rat myocardium. It appears therefore that the reduction in passive K^+ efflux induced by the phenothiazines counteracts to a certain extent the effect of ouabain on the active uptake mechanism.

The mode of action of phenothiazine derivatives as well as of other antifibrillatory agents (HOLLAND 1957; KLEIN *et al.* 1960; CONN & LUCHI 1964; VAN ZWIETEN 1969) is probably related to their effect on the permeability of cardiac muscle membranes. The results presented above lend support to the hypothesis that phenothiazines act mainly by reducing the passive movements of K^+ through the rat heart muscle membranes, even through an effect on the active transport and on the contractile force ("squeezing effect") cannot be completely excluded.

Acknowledgments

I would like to thank Miss Kari Uthus for skilful technical assistance. Financial support from The Norwegian Council on Cardiovascular Diseases is gratefully acknowledged.

I wish to express my thanks to Norfarma and Sandoz for the generous supply of drugs for this investigation.

REFERENCES

Armitage, A. K., J. H. Burn & A. J. Gunning: Ventricular fibrillation and ion transport. *Circulation Res.* 1957, 5, 98–104.

Burn, J. H.: Cardiac fibrillation and ion movements. *Triangle* 1958, 3, 183–190.

Carmeliet, E. E.: The potassium and chloride permeability in Purkinje fibres. In: *Specialized tissues of the heart.* Edited by A. Paes de Carvalho, W. C. de Mello, and B. F. Hoffman. New York, Elsevier, 1961, p. 108–114.

Christensen, J. A., S. S. L. Feng, E. Polly & A. W. Wase: Influence of chlorpromazine on transport of ions into cerebral tissues. *Federation Proc.* 1958, 17, 358.

Conn, H. L. & R. J. Luchi: Cellular basis for regulation of the circulation by digitalis and by quinidine. In: *Structure and function of heart muscle.* Ed. J. R. Evans. American Heart Association Monograph No. 9. 1964, 153–165. New York, The American Heart Association, Inc.

Coraboeuf, E., J. Delahayes & U. Sjøstrand: A comparative study of K^{42} and Na^{24} movements during the cardiac cycle. *Acta physiol. scand.* 1969, 76, 40–48.

Courvoisier, S., J. Fournel, R. Ducrot, M. Kolsky & R. Koetschet: Propriétés pharmacodynamiques du chlorhydrate de chloro-3 (diméthylamino-3'-propyl)- 10 phénothiazine (4560 R. P.). *Arch. int. pharmacodyn.* 1953, 92, 305–361.

Davis, P. W. & T. A. Brody: Inhibition of Na^+ K^+-activated adenosine triphosphatase activity in rat brain by substituted phenothiazines. *Biochem. Pharmacol.* 1966, 15, 703–710.

Dohadwalla, A. N., A. S. Freedberg & E. M. Vaughan Williams· The relevance of β-receptor blockade to ouabain-induced cardiac arrhythmias. *Brit. J. Pharmacol.* 1969, 36, 257–267.

Freeman, A. R. & M. A. Spirtes: Further effects of chlorpromazine in the human erythrocyte membrane. *Biochem. Pharmacol* 1963, 12, 1235–1237.

Glynn, I. M.: The action of cardiac glycosides on ion movements. *Pharmacol Rev.* 1964, 16, 381–407.

Grumbach, L., J. W. Howard & W. I. Merrill. Factors related to the initiation of ventricular fibrillation in the isolated heart: Effect of calcium and potassium. *Circulation Res.* 1954, 2, 452–459.

Grupp, G., G. H. Acheson & A. Charles: Heart rate and paired ventricular stimulation' effects on contractile force and K^+ exchange. *Amer. J. Physiol.* 1967, 212, 607–611.

Guth, P. S. & M A. Spirtes: The phenothiazine tranquilizers: biochemical and biophysical actions. *Int. Rev. Neurobiology* 1964, 7, 231–278.

Hamacher, J. & G. Hildebrandt: Zusammenhänge zwischen chemischer Konstitution und Herz-Kreislauf-Wirkung therapeutisch angewendeter Phenothiazin- und Thiaxanthen-Derivate. *Arzneimittelforsch.* 1964, 9, 977–981.

Holland, W. C.: A possible mechanism of action of quinidine. *Amer. J. Physiol* 1957, 190, 492–494.

Huston, J. R. & G. E. Bell: The effect of thioridazine hydrochloride and chlorpromazine on the electrocardiogram. *J. Amer. Med Ass.* 1966, 198, 16–20.

Klein, R. L. & W. C. Holland: Na^{24} and K^{42} exchange in atrial fibrillation. *Amer. J. Physiol* 1958, 193, 239–243

Klein, R. L., W. C. Holland & B. Tinsley: Quinidine and unidirectional cation fluxes in atria. *Circulation Res.* 1960, 8, 246–252

Landmark, K., A. Glomstein & I. Øye: The effect of thioridazine and promazine on the isolated contracting rat heart. *Acta pharmacol et toxicol.* 1969, 27, 173–182.

Landmark, K. & I. Øye: The action of thioridazine and promazine on biological membranes: Relationship between ATPase inhibition and membrane stabilization. *Acta pharmacol. et toxicol.* 1971, 29, 1–8.

Langslet, A.: Changes in coronary flow and ECG in the isolated perfused rat heart induced by phenothiazine drugs. *Acta pharmacol. et toxicol.* 1969, 27, 183–192.

Langslet, A.: Cardiac effects of antiparkinson drugs, local anesthetics, and tranquilizers. *Eur. J. Pharmacol.* 1970, **9**, 269–275.

Langer, G. A. & A. J. Brady: Potassium in dog ventricular muscle: kinetic studies of distribution and effects of varying frequency of contraction and potassium concentration of perfusate. *Circulation Res.* 1966, **18**, 164–177.

Langer, G. A.: Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractility. *Physiol. Rev.* 1968, **48**, 708–757.

Leestma, J. E. & K. L. Koenig: Sudden death and phenothiazines. *Arch. gen. Psychiat.* 1968, **18**, 137–148.

Madan, B. R. & V. K. Pendse: Antiarrhythmic activity of thioridazine hydrochloride (Mellaril). *Amer. J. Cardiol.* 1963, **11**, 78–81.

Melville, K. I.: Studies on the cardiovascular actions of chlorpromazine: I. Antiadrenergic and antifibrillatory actions. *Arch. int. pharmacodyn.* 1958, **115**, 278–305.

Melville, K. I. & J. V. Drapeau: Studies on the cardiovascular actions of chlorpromazine. II. Effects on cardiac output, coronary flow and heart contractions. *Arch. int. pharmacodyn.* 1958, **115**, 306–325.

Pak, M. J., J. L. Walker jr., E. A. Greene, C. K. Loh & V. Lorber: Measurement of tracer efflux during cardiac cycle. *Amer. J. Physiol.* 1966, **211**, 1455–1460.

Schatzmann, H. J.: Herzglykoside als Hemmstoffe für den aktiven Kalium – Natriumtransport durch die Erythrocytenmembran. *Helv. Physiol. Acta* 1953, **11**, 346–354.

Seeman, P. & J. Weinstein: I. Erythrocyte membrane stabilization by tranquilizers and antihistamines. *Biochem. Pharmacol.* 1966, **15**, 1737–1752.

Sekiya, A. & E. M. Vaughan Williams: The effects of pronethalol, dichloroisoprenaline and disopyramide on the toxicity to the heart of ouabain and anaesthetics. *Brit. J. Pharmacol.* 1963, **21**, 462–472.

Sharma, P. L. & R. B. Arora: Antiarrhythmics. Part XII. Antiarrhythmic activity of some phenothiazine derivatives in experimental cardiac arrhythmias. *Ind. Jour. Med. Res.* 1961, **49**, 1099–1112.

Singh, K. P. & Sharma, U. N.: 10-N substituted phenothiazine derivatives in auricular arrhythmias. *Arch. int. pharmacodyn.* 1969, **177**, 168–178.

Weatherall, M.: Ions and the action of digitalis. *Brit. Heart J.* 1966, **28**, 497–504.

Zweiten, P. A. van: Decrease in ionic permeability of the cell membrane in guineapig atrial tissue by treatment with antifibrillatory agents and hexabarbitone, determined by means of Rb^{86}. *Brit. J. Pharmacol.* 1969, **35**, 103–111.

Øye, I.: The action of adrenaline in cardiac muscle. *Acta physiol. scand.* 1965, **65**, 251–258.

Acta pharmacol. et toxicol. 1971, **29**, 60–64.

From the Department of Pharmacology, University of Göteborg, Sweden

On the Relation between Motor Activity and the Degree of Enzyme Inhibition Following Inhibition of Tyrosine Hydroxylase

By

Torgny H. Svensson and Bertil Waldeck

(*Received June 8, 1970*)

Abstract: The inhibitor of tyrosine hydroxylase, α-methyltyrosine methylester (H44/68), was given intraperitoneally to mice. Following this the motor activity was measured at varying intervals of time and as an index of the activity of tyrosine hydroxylase, the net amounts of ^{3}H-noradrenaline and ^{3}H-dopamine formed in the brain from ^{3}H-tyrosine during one hour were assayed. The hydroxylation of tyrosine was markedly inhibited soon after the administration of the inhibitor while the motor activity declined more slowly. Sixteen hours after the administration of the inhibitor, the motor activity and the amine synthesis were normal and restored to 60 per cent of the normal respectively. The results are discussed in relation to previous data obtained in mice on endogenous brain catecholamine levels after a corresponding injection of H44/68. It is suggested that after inhibition of the synthesis, motor activity depends on the store of catecholamines. However, motor activity appears to be maintained at a low amine level provided that the synthesis is not inhibited.

Key-words: Tyrosine hydroxylase – catecholamines – motor activity.

Inhibition of tyrosine hydroxylase by multiple intraperitoneal injections of α-methyl tyrosine (α-MT) in rats causes behavioural depression and catecholamine depletion with similar time courses (Rech *et al.* 1966). The same tendency was also found by these authors after a single intraperitoneal injection, although the toxicity of the drug made the interpretation of the data difficult. A similar result was found in guinea pigs by Moore (1966), but in this case after a single intraperitoneal injection, the behavioural effects appeared somewhat before the depletion of catecholamines in the brain. The time course of the inhibitory effect on catecholamine synthesis after a single intraperitoneal injection to guinea pigs has also been investigated (Udenfriend *et al.* 1966).

We have now studied the relationships between the time courses of motor activity depression and enzyme inhibition after a single injection of α-MT methylester (H44/68) in mice. The data are discussed in relation to the data on brain catecholamine levels in mice, previously obtained in this laboratory, after a corresponding injection of α-MT methylester.

Methods

All the experiments were performed on female mice (strain N. M. R. I.) weighing about 20 g The ambient temperature was 25°.

The animals were injected with H44/68, 250 mg/kg intraperitoneally. After various time intervals the motor activity was measured during 10 min. immediately after placing the animals in the test cage. These measurements were made with a new instrument, ANIMEX, which has been tested previously (SVENSSON & THIEME 1969). There were three mice in each experimental group

As an indicator of the tyrosine hydroxylase activity the amount of ^{3}H-dopamine (^{3}H-DA) and ^{3}H-noradrenaline (^{3}H-NA) found in the brain was measured one hour after the intravenous administration of 5 μg/kg of ^{3}H-tyrosine. Mice, grouped six by six, received ^{3}H-tyrosine at various time intervals after the intraperitoneal administration of H44/68, 250 mg/kg. The control animals received ^{3}H-tyrosine only. One hour after the injection of ^{3}H-tyrosine the animals were killed and their brains removed and extracted in perchloric acid. The catecholamines were isolated from the extracts by adsorption to alumina and subsequent ion-exchange chromatography on DOWEX 50 (PERSSON & WALDECK 1968; c. f. also SVENSSON & WALDECK 1969).

Results and Discussion

The results, presented as per cent of the respective control values, are shown in fig. 1. After the injection of H44/68 the motor activity decreased and reached a minimum after about 8 hrs. After another 8 hrs the motor activity had returned to normal. When ^{3}H-tyrosine was given 30 min. after H44/68, the amount of ^{3}H-NA found in the brain was approximately 10 per cent of the control value. The yield of ^{3}H-NA was still low 2 hrs after the administration of H44/68, but then started to increase and at 18 hrs was about 60 per cent of the control value. ^{3}H-DA showed changes similar to those of ^{3}H-NA.

Our finding that the maximum reduction in motor activity occurred about 8 hrs after the administration of the tyrosine hydroxylase inhibitor is consistent with the observations on rats, made by RECH *et al.* (1966). The same time lag also exists for a conditioned avoidance response in rats (CORRODI & HANSON 1966). After the inhibition of DA-β-hydroxylase, however, the maximum reduction of motor activity was obtained in half this time (SVENSSON & WAL-

DECK 1969; MOORE 1966). Similarly the brain NA disappeared twice as rapidly after DA-β-hydroxylase inhibition as after tyrosine hydroxylase inhibition (PERSSON & WALDECK 1970).

The synthesis of NA appeared to be inhibited by about 90 per cent within 30 min. after the administration of either of the respective inhibitors (compare the present data with those of SVENSSON & WALDECK (1969)). Sixteen hours after the injection of H44/68, when the synthesis of catecholamines was about 60 per cent of the normal, the motility had recovered completely. CORRODI & HANSON (1966) who studied the catecholamine levels in the mouse (strain N. M. R. I.) brain at various time intervals after H44/68, 250 mg/kg given intraperitoneally. A maximum depletion of amines was obtained between 8 and 24 hrs after the injection.

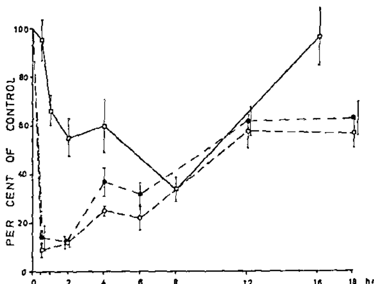


Fig. 1. Effect of the inhibitor of tyrosine hydroxylase, H44/68 on the motor activity of mice and on the formation of ^{3}H-noradrenaline (^{3}H-NA) and ^{3}H-dopamine (^{3}H-DA) from ^{3}H-tyrosine in the mouse brain. H44/68 250 mg/kg was given intraperitoneally and thereafter the motor activity was measured during 10 min. at different intervals of time. Other animals given the same dose of H44/68 received 5 μg/kg ^{3}H-tyrosine at various time intervals. One hour after the administration of ^{3}H-tyrosine, the animals were killed and the ^{3}H-NA and the ^{3}H-DA in the brain determined. The untreated animals and those animals receiving only ^{3}H-tyrosine, respectively, served as controls. The means ± S. E. M. expressed as per cent of the respective control values are shown For the motor activity these were 501 ± 33 counts, for ^{3}H-NA 25.8 ± 3.2 and for ^{3}H-DA 48.0 ± 4.4 fmoles per g. The means are based on 4–10 determinations. 1 fmole = 10^{-15} moles.

——□—— motor activity
– – ○ – – ^{3}H-NA
–·–●–·– ^{3}H-DA

Thus there appears to be no simple correlation between motor activity and catecholamine levels in the brain. It would be reasonable to assume, however, that there is an association between motor activity and the availability of catecholamines in the brain. When the synthesis is blocked, catecholamines are mobilized from the storage granules of the neurons. On the other hand, an adequate transmitter function can be maintained at a low amine level provided that synthesis remains intact. It is interesting to note that KOPIN *et al.* (1968) found that newly synthesized NA was more easily released by nerve stimulation than NA that had been stored for some time.

A tendency for an earlier recovery of the motor activity than of the amine levels in the brain can also be observed in the experiments of RECH *et al.* (1966), using oral administration of α-methyl tyrosine.

It has been proposed that the sedation seen after a single dose of α-MT given parenterally to rats may partly be due to its toxic effects (RECH *et al.* 1966). In our experiments with H44/68 in mice, however, no toxic manifestations were observed and 16 hrs after the injection all the animals had recovered. MOORE *et al.* (1967) found that after a single injection of α-MT given parenterally to rats, the animals which showed toxic symptoms did not recover from their behavioural depression and all the animals died. This discrepancy may be due to differences between the solution of α-MT and its methylester with regard to toxicity (c. f. CORRODI & HANSON 1966). A species difference however, appears equally likely.

In a previous study using a DA-β-hydroxylase inhibitor (SVENSSON & WALDECK 1969) a role of brain NA in the control of motor activity was suggested. The results in the present study using a tyrosine hydroxylase inhibitor are compatible with this view, even though it seems likely that an effect on DA neurons plays a contributary role in the present experiments.

Acknowledgements

This work was supported by the Swedish Medical Research Council (B-70-14X-155-06B) and the Medical Faculty, University of Göteborg. For a generous supply of H44/68 we are indebted to Hässle Ltd., Sweden. The skilful technical assistance of Mrs. Lena Löfberg is gratefully acknowledged.

REFERENCES

Corrodi, H. & L. C. F. Hanson: Central effects of an inhibitor of tyrosine hydroxylase. *Psychopharmacologia* (Berl.) 1966, 10, 116–125.

Kopin, I. J., G. R. Breese, K. R. Krauss & V. K. Weise: Selective release of newly synthesized norepinephrine from the cat spleen during sympathetic nerve stimulation. *J. Pharmacol. Exp. Ther.* 1968, 161, 271–278.

Moore, K. E.: Effects of α-methyltyrosine on brain catecholamines and conditioned behaviour in guinea pigs. *Life Sci.* 1966, **5**, 55–65.

Moore, K. E., P. F. Wright & J. K. Bert: Toxicologic studies with α-methyl tyrosine, an inhibitor of tyrosine hydroxylase. *J. Pharmacol. Exp. Ther.* 1967, **155**, 506–515.

Persson, T. & B. Waldeck: The use of ^{3}H-DOPA for studying cerebral catecholamine metabolism. *Acta pharmacol. et toxicol.* 1968, **26**, 363–372.

Persson, T. & B. Waldeck: Some problems encountered in attempting to estimate catecholamine turnover using labelled tyrosine. *J. Pharm. Pharmacol.* 1970, **22**, 473–478.

Rech, R. H., H. K. Borys & K. E. Moore: Alterations in behaviour and brain catecholamine levels in rats treated with α-methyltyrosine. *J. Pharmacol. Exp. Ther.* 1966, **153**, 412–419.

Svensson, T. H. & Thieme: An investigation of a new instrument to measure motor activity of small animals. *Psychopharmacologia (Berl.)* 1969, **14**, 157–163.

Svensson, T. H. & B. Waldeck: On the significance of central noradrenaline for motor activity: Experiments with a new dopamine β-hydroxylase inhibitor. *European J. Pharmacol.* 1969, **7**, 278–282.

Udenfriend, S., P Zaltzman-Nirenberg, R. Gordon & S. Spector: Evaluation of the biochemical effects produced *in vivo* by inhibitors of the three enzymes involved in norepinephrine biosynthesis *Mol. Pharmacol.* 1966, **2**, 95–105.

Acta pharmacol. et toxicol. 1971, 29, 65–74.

From the Department of Pharmacology (Professor C. G. Schmiterlöw, M. D.),
Royal Veterinary College, Stockholm, Sweden

The Distribution of ^{14}C-4-Mestranol in Mice

By

Lars-Erik Appelgren and Roger Karlsson

(Received May 25, 1970)

Abstract: Whole body autoradiography of mice 2, 20, 60 and 240 min. after intravenous injection of ^{14}C-mestranol showed a rapid and high accumulation of mestranol and/or its metabolites in the adrenal cortex, brown fat and the nervous system. A rather high uptake was also registered in the walls of the follicles and in the corpora lutea. Thin-layer chromatography of methylene chloride extracts indicated demethylation of ^{14}C-mestranol in the body since labelled substance(s) with the same chromatographic behaviour as ethynyloestradiol was/were found in the liver, brown and body fat and in the combined extract from ovaries and uterus.

Key-words: ^{14}C-mestranol-metabolism – distribution.

The cyclopentyl ether derivative of ethynyloestradiol, quinestrol, which has very long lasting oestrogenic effects is known to be concentrated in the body fat and brain (MELI *et al.* 1963). The mother substance, ethynyloestradiol, on the other hand has not been shown to accumulate in these sites (STEINETZ *et al.* 1967). In addition to ethynyloestradiol its methoxy derivative, mestranol, is widely used as an ingredient in contraceptive pills of the combination type (cf. DICZFALUSY 1968). The antifertility effects of exogenous oestrogens have also been used clinically after accidental matings in dogs and mestranol in a single oral dose has been shown to produce abortion (KENNELLY 1969).

Considering the relatively great use of mestranol it was of interest to investigate whether this derivative of ethynyloestradiol was also localized in the brain and the body fat. Whole body autoradiography as well as impulse counting was made using mice injected intravenously with ^{14}C-4-mestranol. In order to find out to what extent the radioactivity represented mestranol and/or its metabolites in the body, some chromatographic studies were made. A preliminary report of the autoradiographic part of this work has been presented (APPELGREN & KARLSSON 1969).

Materials and Methods

^{14}C-4-Mestranol with a specific activity of 45.2 mci/mM was obtained from New England Nuclear Corp., Boston, Mass., USA. Thirteen female albino mice (NMRI), one of them pregnant in a late stage of gestation, were used. The average weight of the non-pregnant mice was 22 g. Each animal received a single dose of 1.25 μci corresponding to 0.009 mg mestranol. This dose was dissolved in 25 μl ethanol and injected intravenously into a tail vein (cf. APPELGREN 1967).

Whole body autoradiography.

Five of the animals were used for whole body autoradiography and were sacrificed after 2 min. (2 animals), 20 min. (1 pregnant and 1 non-pregnant female mouse) and 60 min. respectively. After freezing the animals were sectioned and autoradiographed according to the Ullberg method (ULLBERG 1954 and 1958). In order to avoid the artefacts observed in the autoradiograms of fat soluble substances due to the melting of fat at room temperature, all dark room work, except developing, was carried out at −10° (cf. APPELGREN 1967). The exposure time varied from 3 to 5 months.

Impulse counting.

Four female mice sacrificed after 2, 20 and 60 min. and 4 hrs were used for impulse counting. The following samples were taken: blood, uterus, liver, body fat, brown fat, brain, adrenal and ovary. The tissues were immediately weighed and wet ashed with perchloric acid and counted in a liquid scintillation spectrometer (Tricarb 3003, Packard) according to MAHIN & LOFBERG (1966).

Chromatography.

Four female mice sacrificed after 2, 20 and 60 min. and 4 hrs were used for the chromatographic experiments. The following samples were taken: blood, uterus and ovary, liver, body fat, brown fat and brain. The tissues were immediately weighed and after freezing extracted twice with methylene chloride. The methylene chloride extracts were evaporated to dryness with N_2. The residue was dissolved in 1 ml methylene chloride and 25 μl of this material were counted in a liquid scintillation spectrometer after the addition of 10 ml toluol-PPO (5 g PPO/1). Chromatography of the methylene chloride extracts was made on thin-layer silica gel G plates according to STAHL (1967). After preliminary experiments the following developing system was used: ethyl ether-cyclohexane (80 : 20) which separated the reference substances used. These reference substances were mestranol, ethynyloestradiol and oestradiol. Since variations in the Rf-values were seen, the reference substances were run on the same plates as the radioactive tissue extracts, in order to facilitate comparison of the radioactive and reference substances. The chromatograms of the non-radioactive substances were made visible by spraying the plates with sulphuric acid-methanol (1 : 1) and heating them for 20 min. at + 100°.

The radioactivity of the chromatograms was detected with the aid of a radiochromatogram scanner (Packard) and/or autoradiography. The relative concentration of each spot was determined by impulse counting in a liquid scintillation spectrometer (Packard) (cf. APPELGREN 1967) In some cases no radioactivity could be detected in the chromatograms despite an autoradiographic exposure of 4 months.

Results

Whole body autoradiography (fig. 1, 2, 3, and 4).

The most remarkable finding was the rapid and high accumulation of ^{14}C in the adrenal cortex followed by the brown fat and the central nervous system. A rather high uptake was also noticed in different parts of the ovaries and in the liver. The distribution in the various organs will be described in detail below.

The circulatory system. The blood concentration was lower than that of the liver and lung as early as 2 min. after the injection and remained low throughout the experiment. The heart muscle and the large vessels showed a concentration somewhat higher than that of the blood during the first 20 min., but then decreased to the same level as the blood.

The lymphatic organs. No specific uptake could be noticed in the spleen, thymus or in the lymphatic glands.

The adrenal glands. Two minutes after the injection there was a very high concentration of ^{14}C in the adrenal cortex. The radioactivity seemed to be more concentrated in the glomerular and fascicular layers of the adrenal cortex. After 20 min. the concentration had decreased but was still high. One hour after the injection the concentration in the adrenal cortex was low. The adrenal medulla showed very small amounts of radioactivity during the experiment.

The reproductive system. In the ovaries the highest concentrations of ^{14}C were seen in the walls of the follicles and in the corpora lutea 2 and 20 min. after injection. The interstitial parts showed a moderate blackening. The uterus and vagina showed a slight accumulation 2 and 20 min. after injection.

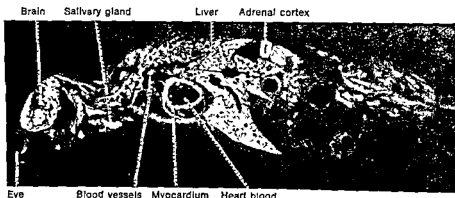


Fig 1. Autoradiogram of a female mouse 2 min. after injection of ^{14}C-4-mestranol. The adrenal cortex, kidney, liver and brain show high uptake of ^{14}C (light areas).



Fig. 2. Detail from a whole body autoradiogram of a female mouse 2 min. after injection of ^{14}C-4-mestranol showing the uptake of ^{14}C in the interstitial parts and follicular walls of the ovary.

The radioactivity seemed to be mainly localized to the endometrium in the uterus. In the pregnant mouse very little radioactivity could be shown in the foetuses or in the placenta.

The nervous system. Two min. after injection there was a high concentration of ^{14}C in the central nervous system, mainly confined to the gray matter.

The spinal ganglia and the peripheral nerves also had rather high concentrations at that time. The central nervous system showed a relatively high concentration of ^{14}C throughout the whole investigation, but there was a change in the distribution from the gray matter towards a higher concentration in the white matter. There was a marked decrease of the radioactivity with time in the spinal ganglia and nerves. The ^{14}C-concentration in the pituitary was slightly lower than that of the brain. A slight accumulation could be noticed in the lens and choroid of the eye 2 min. after injection, but this disappeared in the later stages.



Fig. 3. Autoradiogram of a female mouse 20 min. after injection of ^{14}C-4-mestranol. Note the high uptake of ^{14}C in the brain, brown fat and bronchi.

The digestive system. The teeth showed an intermediate level of radioactivity 2 min. after injection. The highest concentrations were seen in the pulp and the dentine.

The glandular part of the stomach showed distinct localization of ^{14}C 2 min. after injection which had disappeared after 20 min.

The liver as a whole had a high concentration of ^{14}C throughout the experiment, but 2 min. after the injection there was a very high concentration in a narrow zone surrounding the hepatic vessels. This specific pattern could not be found in the later stages.

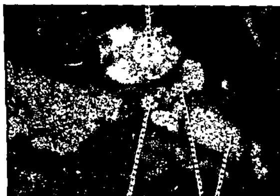


Fig. 4. Detail from a whole body autoradiogram of a female mouse 20 min. after injection of ^{14}C-4-mestranol. Note the high concentration of ^{14}C in the corpora lutea of the ovary.

After 20 min. there was a very marked accumulation of ^{14}C in the bile ducts and in the gall bladder. The intestinal contents showed no radioactivity until after 20 min.; this was probably due to biliary excretion.

The pancreas showed a moderate accumulation of ^{14}C 2 min. after injection but had low amounts of radioactivity in the later stages.

The respiratory system. The nasal mucosa had a moderate and rather constant concentration of ^{14}C during the experiment.

The parenchyma of the lung showed a concentration slightly exceeding that of the blood 2 min. after the injection, but later the concentration was the same as in the blood.

The bronchi showed high concentration of ^{14}C *20 min. after injection, which also remained rather high after 60 min.*

The urinary organs. Two min. after the injection there was a high uptake in the kidney and small amounts of radioactivity in the urinary bladder. After 20 min. the concentration of ^{14}C was low in the kidney, but fairly high in the ureters and the urinary bladder. After 1 hr, there was a low level of radioactivity in the urinary organs.

The skin. The concentration of ^{14}C in the skin and subcutis never exceeded that of the blood.

The hard tissues. In the bone no ^{14}C was registered. In the bone marrow the ^{14}C concentration was rather high after 2 min. but showed a rapid decrease.

Muscles. The skeletal muscles showed a moderate uptake 2 min. after injection but decreased with time.

Brown fat. The brown fat showed a *high concentration of* ^{14}C *2 minutes after injection. A rather high concentration was maintained throughout the experiment in contrast to the body fat which showed an initially low but somewhat increasing* ^{14}C-concentration.

Impulse counting.

The radioactivity in the different organs was calculated as % g dose per g tissue and is presented in fig. 6. The results from the quantitation of the methylene chloride extracts used for chromatography agreed fairly well with the values obtained when the whole tissue was wet ashed and scintillated, except in the liver. The liver probably contained rather large amounts of ^{14}C which were not extracted with methylene chloride.

There was good agreement between the autoradiographic study and the quantitative measurements except in a few cases. When the radioactivity was localized in certain areas within an organ, as was shown with autoradiography e. g. in the cortex of the adrenal and in the white matter of the brain, the quantitative measurements were not up to the concentration per g of these specific tissues.

Chromatography (fig. 5).

Chromatography of the methylene chloride extracts from the *blood* and the *brain* 2–60 min. after the injection revealed only one major spot with an Rf-value corresponding to mestranol.

The *brown fat* showed one major metabolite corresponding to ethynyloestradiol 2 and 60 min. after injection. In the *body fat* only one metabolite with an Rf-value corresponding to that of ethynyloestradiol was registered 20 and 60 min. after injection.

In the liver several metabolites were registered, two of them with Rf-values corresponding to mestranol and ethynyloestradiol respectively. The major portion of the metabolites in the liver extracts were however more polar than mestranol and ethynyloestradiol.

In the combined extract from *uterus* and *ovaries* most of the radioactivity had an Rf-value corresponding to mestranol and a very small amount corresponding to ethynyloestradiol 2 and 20 min. after injection.

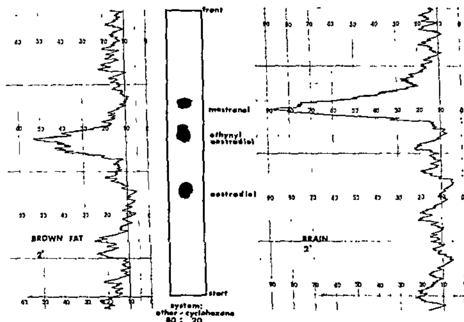


Fig. 5. Diagrams of radioactive thin-layer chromatograms scanned in a radiochromatogram scanner. Tissue extracts from brown fat and brain from an animal 2 min. after injection of 14C-4-mestranol were chromatographed in ether-cyclohexane (80 : 20). In the brown fat the main part of the radioactivity corresponded to ethynyloestradiol and in the brain to mestranol.

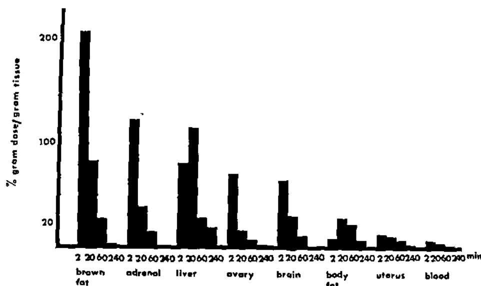


Fig. 6. Radioactivity in brown fat, adrenals, livers, ovaries, brains, body fat, uteri and blood at different intervals after intravenous injection of ^{14}C-4-mestranol expressed as % gram dose per gram tissue.

Discussion

The whole body autoradiographic distribution of ^{14}C-mestranol agrees well with the distribution pattern for natural oestrogens as described by ULLBERG & BENGTSSON (1963) except in a few cases. Mestranol accumulated to a higher extent in the brown and body fat and in the brain than did the natural oestrogens. This accumulation of ^{14}C-mestranol in the brain and fat was however observed only shortly after the injection contrary to what has been shown previously for quinestrol (MELI *et al.* 1963; 1965; 1968).

The chromatographic studies indicated that most of the radioactivity in the blood and brain corresponded to mestranol a finding that agrees with the experiments of JENSEN *et al.* (1966) who found that the blood contained about 70 % labelled mestranol 15 min. after subcutaneous injection of ^{3}H-mestranol to young rats. JENSEN *et al.* (1966) suggested that in order to act as an oestrogen, mestranol must first be demethylated to furnish ethynyloestradiol, which is taken up and retained by receptors in target tissues, such as the uterus. In the combined extracts from uterus and ovaries in the present investigation, there were small amounts of radioactivity that might be connected with ethynyloestradiol at short time intervals after the injection. It was suggested that the main site of demethylation was the liver (JENSEN *et al.* 1966) but this investigation also indicated a similar function for brown fat

in the mouse, since most of the high amount of radioactivity present in this tissue behaved chromatographically as ethynyloestradiol.

In the body fat the radioactivity was only connected with ethynyloestradiol and since the radioactivity increased with time it might be suggested that the demethylation of mestranol occurred elsewhere.

In the ovary the radioactivity was mainly distributed to the follicle walls and the corpora lutea. Whether the radioactivity was confined only to the granulosa cell layers could not be determined from the present whole body autoradiograms. The granulosa cell layers have been shown to be target organs for ^{3}H-oestradiol by ULLBERG & BENGTSSON (1963) and later by STUMPF (1969).

The accumulation of ^{14}C-mestranol and/or metabolites in the corpora lutea can be correlated to the finding of radioactivity in these structures after the injection of ^{14}C-diethylstilboestrol (BENGTSSON & ULLBERG 1963).

Acknowledgements

The authors are indebted to Knut and Alice Wallenbergs Stiftelse for general equipment for isotope research work. We wish to thank Mrs. E. Stålfelt and Miss K. Ledin for their skilled technical assistance.

REFERENCES

Appelgren, L.-E.: Sites of steroid hormone formation. Autoradiographic studies using labelled precursors. *Acta physiol. scand.* 1967, Suppl. 301, 1–108.

Appelgren, L.-E. & R. Karlsson: The distribution of labelled mestranol in mice. *Acta endocr.* (Kbh.) 1969, Suppl. 138, 84.

Bengtsson, G. & S. Ullberg: The autoradiographic distribution pattern after administration of diethylstilboestrol compared with that of natural oestrogens. *Acta endocr. (Kbh.)* 1963, 43, 561–570.

Diczfalusy, E.: Mode of action of contraceptive drugs *Am. J. Obst. Gynec.* 1968, 100, 136–163.

Jensen, E. V., H. I. Jacobsen, J. W. Flesher, N. N. Saha, G. N. Gupta, S. Smith, V. Colucci, D. Shiplacoff, H. G. Neumann, E. R. DeSombre & P. W. Jungblut: Estrogen receptors in target tissues. In: *Steroid dynamics.* (Eds. G. Pincus, T. Nakao & J. F. Tait), Academic Press, N. Y. and London, 1966, 133–157

Kenelly, J. K.: The effect of mestranol on canine reproduction. *Biol. Reprod.* 1969, 1, 282–288.

Mahin, D. T & R. T. Lofberg: A simplified method of sample preparation for determination of tritium, carbon -14 or sulphur-35 in blood or tissue by liquid scintillation counting. *Analyt. Biochem.* 1966, 16, 500–509.

Meli, A, A. Wolff & W. L. Honrath: The mechanism by which 3- etherification with cyclopentyl alcohol enhances the oral activity of ethynylestradiol. *Steroids* 1963, 2, 417–424.

Meli, A., B. G. Steinetz, V. L. Beach, A. Wolff & T. Giannina: Biological and chromatographic evidence for the storage of ethynylestradiol -3-cyclopentyl ether in rat brain. *Proc. Soc. exp Biol. Med.* 1965, 119, 602–606.

Meli, A., B. G. Steinetz, T. Giannina, D. I. Cargill & J. P. Manning: Fat storage, deetherification and elimination of quinestrol in the rat as influenced by route of administration. *Proc. Soc. exp. Biol. Med.* 1968, **127**, 1042–2048.

Stahl, E.: *Dünnschicht – Chromatographie*, ed. 2. Springer Verlag, Berlin-Heidelberg-New York, *1967, p. 53.*

Steinetz, B. G., A. Meli, T. Giannina, V. L. Beach & J. P. Manning: Distribution of radioactivity in body fat and organs of rats treated with labeled quinestrol, ethynylestradiol or 17β-estradiol. *Proc. soc. exp. Biol. Med.* 1967, **124**, 111–117.

Stumpf, W. E.: Nuclear concentration of ^{3}*H-estradiol in target tissues. Dry-mount* autoradiography of vagina, oviduct, ovary, testis, mammary tumor, liver and adrenal. *Endocrinology* 1969, **85**, 31–37.

Ullberg, S.: Studies on the distribution and fate of S^{35}-labelled benzylpenicillin in the body. *Acta radiol. 1954, Suppl. 118, 1–110.*

Ullberg, S.: Autoradiographic studies on the distribution of labelled drugs in the body. *Second U. N. Int. Conf. Peaceful uses of Atomic Energy* 1958, **24**, 248–254.

Ullberg, S. & G. Bengtsson: Autoradiographic distribution studies with natural oestrogens. *Acta endocr. (Kbh.) 1963, **43**, 75–86.*

Acta pharmacol. et toxicol. 1971, 29, 75–80.

From the Industrial Toxicology Research Centre,
Lucknow, U. P. India

Cellular Changes Induced by Manganese in the Rat Testis – Preliminary Results

By

Satya V. Chandra

(Received February 27, 1970)

Abstract: Testicular damage was produced in rats by the intraperitoneal administration of manganese chloride (8 mg/kg) daily for a period of 180 days. Histopathological examination performed at 120 days showed oedema and congestion, at 150 days, degeneration of spermatids in about one third of the seminiferous tubules and subsequently at 180 days, about 50 per cent of seminiferous tubules were degenerated in all the animals. It is suggested that manganese possibly has a specific direct action on the spermatogenic epithelium.

Key-words: Manganese – toxicity – rat testis.

The toxic effects of manganese on the central nervous system and other vital organs such as the liver and kidney have been studied (HURST & HURST 1928; OETTINGEN 1935; PENTSCHEW *et al.* 1963) but its action on the testicular tissue has not been investigated. A few reports are, however, available of the effect on the testis of other metals like zinc and cadmium which are physicochemically similar to manganese. Chronic nutritional deficiency of zinc and experimentally administered cadmium has been reported to produce testicular damage (ELCOATE *et al.* 1955; PARIZEK 1957). It was, therefore, of interest to study the effects of parenterally administered manganese chloride on the testicular tissue of the rat.

Materials and Methods

Male albino rats of average weight of 120 g from I. T. R. C. Colony were used. They were fed a standard diet* and allowed water ad libitum.

---

* The contents of calcium and phosphorus in this diet for one rat per day were 34.15 mg and 80.67 mg respectively. The calcium phosphorus ratio was 1:2.36, while only traces of manganese were present.

Ninety rats were divided into two groups. Group I consisted of 60 animals which *were given manganese chloride (8 mg/kg) intraperitoneally daily. This dose was calculated* on the basis of the experiments performed by HURST & HURST (1928) and of previous work reported from this laboratory (CHANDRA & SRIVASTAVA 1970). Group II *consisted of 30 rats which were used as controls and received no treatment.*

Ten animals from group I and five from group II were sacrificed at intervals of 30 days up to a period of 180 days. However the numbers of animals sacrificed from group I at 150 and 180 days were six and seven respectively, due to the death of seven experimental rats. The dead and sacrificed animals were autopsied. The testes were removed, bisected, weighed and immediately placed in buffered neutral formalin. The tissue was dehydrated in graded alcohols and after complete dehydration embedded in paraffin. Sections were cut at 5 μ and stained with haematoxylin and eosin.

Results

Gross appearance.

The testes from the manganese treated and control group were light pink in colour at all the periods of the sacrifice.

Livers from manganese treated animals showed mild congestion. The remainder of the viscera did not reveal any gross pathological change in either of the groups.

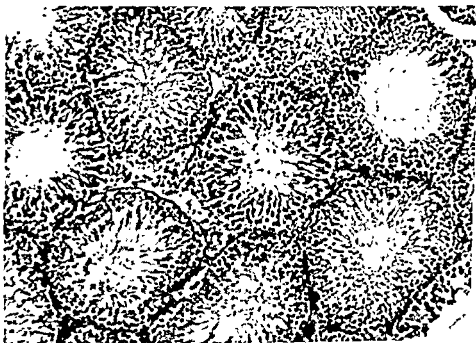


Fig. 1. Testis of normal rat showing orderly arrangement of germ cells in the seminiferous tubules and interstitial tissue. Haematoxylin and eosin. Magnification × 285.

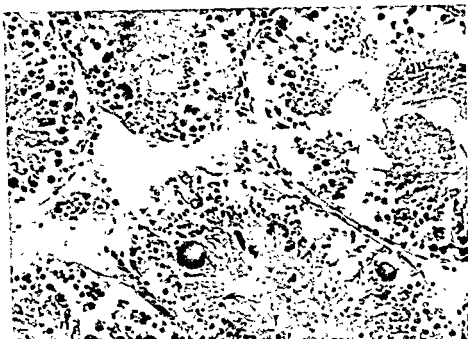


Fig. 2. Testis of rat injected with manganese chloride (8 mg/kg) daily for 150 days. Seminiferous tubules show degenerative changes and multinucleate cells. Haematoxylin and eosin. Magnification × 350.

Microscopic examination of testes.

The histological pattern of the control group (normal) testis showed an orderly arrangement of the germ cells in the seminiferous tubules and sparsely cellular strands of interstitial cells of Leydig throughout the experiment (fig. 1).

Testes of the treated group examined up to 90 days had a histological appearance comparable to the normal structure.

The earliest changes appeared at 120 days with the development of oedema of the interstitial tissue and vascular engorgement.

At 150 days obvious degeneration was present in an appreciable number of spermatids, pyknosis being prominent in the nuclei. About one third of the seminiferous tubules were affected. Dissociation of altered seminiferous epithelial cells with resultant desqumation was noted in a few degenerated tubules, and eosinophilic material was present in the lumen of degenerated tubules which appeared in groups among relatively normal tubules. The number of spermatocytes was considerably reduced in the tubules, and depletion was also noted in the number of spermatids in few of the specimens.

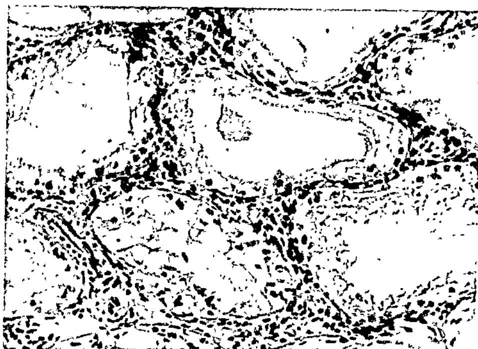


Fig. 3. Testis of rat injected with manganese chloride (8 mg/kg) daily for 180 days. Seminiferous tubules show marked depletion of germinal epithelium, only Sertoli cells and few spermatogonia are seen. Haematoxylin and eosin. Magnification × 350.

There were a few multinucleate cells in the lumen of the seminiferous tubules (fig. 2).

At 180 days about 50 per cent of the tubules showed a marked loss in the number of spermatocytes. The spermatids were also markedly depleted in the degenerated tubules. Numerous multinucleate cells, degenerating spermatocytes and spermatids were found entangled in the cytoplasm of Sertoli cells. In the testes of two rats, groups of seminiferous tubules were depleted and were lined by a single layer of cells consisting of Sertoli cells and a few spermatogonia (fig. 3). The basement membrane of the tubules did not show any thickening. In the testes of three rats, groups of degenerated tubules were partially collapsed and their lumen contained eosinophilic material. Leydig and other cells of the interstitial tissue were unaffected except in few rats where there was a slight increase in the interstitial tissue with an abundance of Leydig cells. Blood vessels appeared normal at 150 and 180 days. The tunica albuginea did not show any change.

The microscopic changes in the brain in similar type of experiment have been reported by CHANDRA & SRIVASTAVA (1970). The remaining viscera were not examined for microscopic changes.

Discussion

Testes have a high cell turnover during the reproductive period and are specially vulnerable to a wide variety of stimuli (ADAMSTONE & SPECTOR 1950; KAUFMAN *et al.* 1956; STEINBERGER & DIXON 1959; DROBECK & COULSTON 1962; PARIZEK 1957; ELCOATE *et al.* 1955). The present experiments demonstrate that the testes are also highly sensitive to the action of manganese. Daily intraperitoneal administration of manganese chloride to rats resulted in an appreciable damage to the seminiferous tubules at 150 days and subsequently at 180 days and in all the animals about 50 per cent of the tubules were degenerated.

In our experiments the damaging effect of manganese on the spermatogenic epithelium was slow and gradual. This may be due to the small doses of manganese used and possibly larger concentrations or more prolonged exposure may lead to a more extensive damage to the testes. Cadmium which is physicochemically similar to manganese, produces extensive damage to the testicular tissue in much smaller doses and within 48 hours (MEEK 1959). With cadmium it is not yet certain whether the damage to the seminiferous epithelium is direct or secondary to the changes in the vascular endothelium (GUNN *et al.* 1961). The present experiments demonstrate that manganese does not cause any vascular lesions. There is no evidence of interstitial haemorrhage or any thrombosis. It is possible that manganese has some specific direct action on the spermatogenic epithelium in rats. The mechanism of action of these two metals require further investigation.

PARIZEK (1957) in his experiment administered zinc together with cadmium and noted prevention of testicular lesions. He suggested that cadmium disturbed the normal zinc metabolism and that its replacement helped in the prevention of the testicular lesions. The same mechanism may also be true for manganese and this needs further investigation.

A large number of chemical compounds (phytic acid, acetoglycerides, naphthalene, trans-aconitic acid etc.) are also known to damage the testis in experimental animals (RIBELIN 1963). The changes due to most of these compounds are mainly confined to the spermatogenic epithelium. It is not known what common denominator links these compounds, or whether there is a common linkage. How far these unknown factors are operative in manganese toxicity need further work. Our results, however, show that the testes are highly sensitive to the deleterious effect of manganese.

Acknowledgement

Thanks are due to Dr. S. H. Zaidi, Director, Industrial Toxicology Research Centre, for his valuable suggestions and guidance throughout the course of the experiments. I wish to thank Shri R. S. Srivastava for technical assistance and Shri Musleh Ahmad for the microphotography.

REFERENCES

Adamstone, F. B. & H. Spector: Tryptophane deficiency in the rat. Histological changes induced by forced feeding of an acid hydrolyzed casein diet. *Arch. Path.* 1950, **49**, 173–184.

Chandra, Satya V. & S. P. Srivastava: Experimental production of early brain lesions in rats by parenteral administration of manganese chloride. *Acta pharmacol. et toxicol.* 1970, 28, 177–183.

Drobeck, H. P. & F. Coulston: Inhibition and recovery of spermatogenesis in rats, monkeys and dogs medicated with bis (dichloroacetyl) diamines. *Exp. Molec. Path.* 1962, **1**, 251. Quoted from *Arch. Path.* 1967, **84**, 376–392.

Elcoate, P. V., M. I. Fischer, C. A. Mawson & M. J. Millar: The effect of zinc deficiency on the male genital system. *J. Physiol.* 1955, **129**, 53–54.

Gunn, A. Samuel, T. C. Gould, W. A. Anderson & Coral Gables: Zinc protection against cadmium injury to rat testis. *Arch. Path.* 1961, 71, 274–281.

Hurst, E. W. & P. E. Hurst: The etiology of hepatolenticular degeneration: Experimental liver cirrhosis: Poisoning with manganese, chloroform, phenylhydrazine, bile and guanidin. *J. Path. Bact.* 1928, 31, 303–342.

Kaufman, N., J. V. Klavins & T. D. Kinney: Testicular damage following ethionine administration. *Amer. J. Path.* 1956, **32**, 105–116.

Meek, E. S.: Cellular changes induced by cadmium in mouse testis and liver. *Brit. J. Exp. Path.* 1959, **40**, 503–506.

Oettingen, Von, W. F.: Manganese: it's distribution, pharmacology and health hazards. *Physiol Reviews.* 1935, **15**, 175–201.

Parizek, J.: The destructive effect of cadmium ion on testicular tissue and its prevention by zinc. *J. Endocrinol.* 1957, **15**, 56–63

Pentschew, A., F. F. Ebner & R. M. Kovatch: Experimental manganese encephalopathy in monkeys. *J. Neuropath. & Exp. Neurology* 1963, 22, 488–499.

Ribelin, W. E.: Atrophy of rat testis as index of chemical toxicity. *Arch. Path.* 1963, **75**, 229–235.

Steinberger, E. & W. J. Dixon: Some observations on the effect of heat on the testicular germins epithelium. *Fertil. Steril.* 1959, **10**, 578. Quoted from *Arch. Path.* 1967, **84**, 376–392.

Acta pharmacol. et toxicol. 1971, 29, 81–86.

From the Department of Microbiology, Dental Faculty,
University of Oslo, Norway

The Herbicide 2,4-Dichlorophenoxyacetic Acid I: Effects on L Cells

By

Jan Kolberg, Kristen Helgeland, Jon Jonsen and Olav Tjeltveit

(Received May 19, 1970)

Abstract: The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on L 929 cells in monolayer cultures has been studied. It was found that 2,4-D in the range of 50 to 500 µg/ml had a dose dependent inhibitory effect on cell growth. With 350 and 500 µg/ml complete inhibition of growth occurred after about 24 hrs of incubation. On removal of 2,4-D a rapid resumption of cell multiplication took place. This occurred even after exposure to 500 µg 2,4-D/ml for 12 days. In the presence of 250 to 500 µg/ml, vacuoles, which stained with lipid soluble dyes, appeared in the cytoplasm. On prolonged incubation with the herbicide, these vacuoles disappeared.

Key-words: Herbicide – L cells.

2,4-dichlorophenoxyacetic acid (2,4-D) belongs to the phenoxy group of herbicides which at low concentrations induces growth responses similar to the plant hormone auxin. At higher concentrations of these herbicides an excessive, uncontrolled growth leading to the death of the plant is observed. This is believed to be based on an abnormal metabolism of RNA (HANSON & SLIFE 1969).

2,4-D is probably used to a greater extent than any other herbicide, and descriptions of its toxicity and hazards to man, domestic animals and wildlife have been summarized by WAY (1969). The acute toxicity following oral administration appears to be moderate. The LD50 values found for a number of experimental animals range between 300 and 800 mg 2,4-D/kg body weight. In a recent, yet unpublished, study commissioned by the National Cancer Institute (U. S. A.) this herbicide was labelled as being a potentially teratogenic compound needing further study (*Science* 1969, *166*, 977 news and comment).

Since 2,4-D might be a teratogenic agent, studies to elucidate the mechanism of action on mammalian cells is of importance. For this purpose, cell cultures provide a suitable tool. The only study in this field has recently been

made by LI & JORDAN (1969) who found a transient growth inhibition in suspension cultures of L cells exposed to 5 and 10 µg/ml of an ester of 2,4-D.

The present paper describes the effect of different concentrations of 2,4-D Na-K salt on the growth and morphology of L 929 cells in monolayer cultures.

Materials and Methods

Cell culture techniques.

Monolayers of mouse fibroblasts, strain L 929 (SANFORD *et al.* 1948) were used. The cultures were grown in Eagle's Minimum Essential Medium supplemented with 10 % calf serum, streptomycin and penicillin. Plastic petri dishes, 60 × 15 mm (Falcon Plastic Inc.) were seeded with 0.4×10^6 cells suspended in 5 ml medium and incubated at 37° in a humidified atmosphere of CO_2 in air. After incubation for 24 hrs, the medium was replaced by test media containing 2,4-D. The media were renewed every third day.

In reversal experiments the 2,4-D containing medium was removed and, before adding the control medium, the cell layers were washed twice with 2 ml of the latter medium.

For growth measurements, the cultures were trypsinized and counted in a *Bürker* haemocytometer. Each point on the growth curves represents the mean of the counts from 2 cultures.

Cytochemical analysis of lipids were carried out with Oil Red 0 in 100 % isopropanol or Sudan Black B in 70 % ethanol and counterstaining with Harris haematoxylin and neutral red respectively. The cells were not fixed before staining.

Cultures cultivated in Sykes and Moore chambers, volume 0.7 ml (SYKES & MOORE 1960) were photographed at 2 frames/min. by means of a Reichert inverted phase-contrast microscope with a 16 mm Beaulieu camera loaded with Kodachrome II A film. To reverse the effect of 2,4-D, the chambers were perfused with 2.5 ml control medium.

Herbicide.

A stock solution containing 25 mg 2,4-D/ml was prepared by dissolving the acid (Eastman Organic Chemicals) in aqueous NaOH-KOH (0.1 N : 0.1 N) and adjusting the pH to 7.3 with HCl. The solution was sterilized by filtration through a 0.22 µ Millipore membrane filter.

Results

On exposure of L 929 cells in monolayer cultures to 2,4-D in concentrations from 50 to 500 µg/ml, a dose dependent inhibition of growth was found (fig. 1). Complete inhibition was found after 24 hrs in the presence of 350 and 500 µg 2,4-D/ml. Cytopathogenic changes such as rounding up and detachment of the cells were not seen, however, in the treated cultures.

When 2,4-D was removed from the cultures after incubation in the presence of 500 µg/ml for 3 or 12 days, inhibition of growth ceased (fig. 2). On removal of 2,4-D after exposure for 3 days there was a rapid increase in the number of cells concomitant with a disappearence of the vacuoles, whereas after 12 days there was a lag period of about 24 hrs before cell

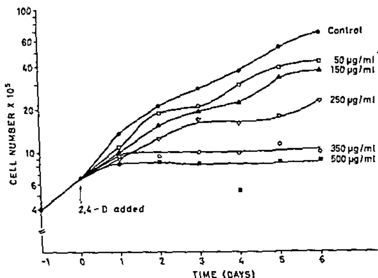


Fig. 1. The effect of various concentrations of 2,4-D on growth of L 929 cells.

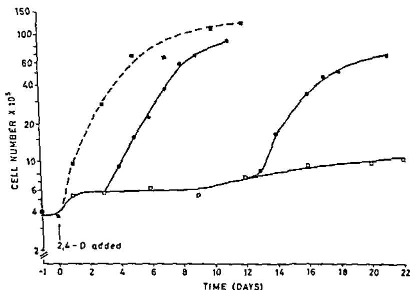


Fig. 2. Resumption of cell growth after removal of 2,4-D.
■ - - - - ■ untreated cultures.
□——□ cultures containing 500 µg/ml.
●——● cultures from which 2,4-D has been removed after 3 or 12 days of treatment.



Fig 3. L 929 cells photographed under phase-contrast microscope.
a. control cells.
b. cells treated with 500 μg 2,4-D/ml for 30 hrs.
Magnification × 600.

multiplication started. This experiment also shows that the cultures on prolonged incubation in the presence of 500 μg 2,4-D/ml exhibited an adaption to this compound and some growth occurred.

In the presence of 350 and 500 μg/ml, vacuoles of uniform size (fig. 3), which stained with Oil Red O and Sudan Black B, appeared in the cytoplasm after about 3 hrs of incubation, and their number increased during the next 21 hrs. On further incubation, the vacuoles disappeared, and after treatment for 4–5 days the cells were free from lipid-containing particles. At 250 μg/ml, vacuoles were observed after 24 hrs, but they disappeared within 2–3 days of incubation. No accumulation of vacuoles was found in the cultures treated with 50 and 150 μg/ml.

The observations made in the previous experiments were confirmed by time-lapse cinemicrography. During 33 hrs in the presence of 500 μg 2,4-D/ml no cell division was seen, and vacuoles were formed. When 2,4-D was removed a rapid resumption of cell growth took place, and the vacuoles disappeared within 22 hrs without being extruded intact into the medium. The cultures were followed for 32 hrs after removal of 2,4-D and there was no indication of a synchronizing effect from the treatment with this compound. No obvious effects of 2,4-D on pinocytosis and the undulating activity of the cell membranes were found.

Discussion

A concentration dependent inhibition of growth was found, when monolayers of L cells were exposed to 2,4-D. The inhibition was evident after 24 hrs, and with 350 and 500 μg 2,4-D/ml no net increase in the numbers of cells was found during the next 5 days of incubation. Some growth occurred, however, during the first 24 hrs in the presence of these two high concentrations which might indicate that 2,4-D was selectively toxic to a particular stage in the cell cycle. As this inhibitory effect of 2,4-D was readily reversible, a synchronizing effect would be expected if such a mechanism was operating. When cultures treated with 500 μg 2,4-D/ml for 33 hrs were transferred to control medium and examined with time-lapse cinemicrography, no burst of synchronized cell division was seen. This makes the above explanation unlikely.

The rapid resumption of cell growth on removal of 2,4-D after treatment for 3 and 12 days seems to exclude a state of unbalanced growth. This condition usually results in cell death on prolonged exposure to the inhibitor (MUELLER 1969).

During a period of 21 days, the L cells exhibited an adaption to growth in the presence of 500 μg/ml. This may be due to a selection of resistent cells and/or a prolongation of the generation time.

It has previously been shown that changes in the extracellular environment cause accumulation of lipid-containing particles in cultured mammalian cells. These changes include a decrease in the extracellular pH, the addition to the medium of phenol or aliphatic polyalcohols, the replacement of horse serum by rabbit or human serum (MACKENZIE *et al.* 1967), and the addition of an unsaturated fatty acid such as oleate, linoleate and linolenate (MOSKOWITZ 1967). These vacuoles, like those induced by 2,4-D, stained with lipid soluble dyes and exhibited little or no tendency to fuse. However, these investigators found no growth inhibition with lipogenic factors in the medium, except for some growth retardation caused by the polyalcohols (MACKENZIE *et al.* 1968).

On continued incubation with 2,4-D, the vacuoles disappeared. MOSKOWITZ (1967) observed a similar phenomenon in oleate-treated L cells. He suggested that the particles might have been extruded intact into the medium by a secretory mechanism or, alternatively, that an intracytoplasmic hydrolysis might account for the breakdown and removal of the lipid particles. In favour of the hydrolytic mechanism, he demonstrated an increased lipase activity in steatotic cells. Whether a similar mechanism operates in our cells has not been investigated.

We found a transient lipid accumulation in 2,4-D treated cells and a release of growth after changing to the control medium. These findings suggest that the occurrence of lipid vacuoles in cell cultures is not necessarily indicative of degeneration.

REFERENCES

Hanson, J. B. & F. W. Slife: Role of RNA metabolism in the action of auxin-herbicides. *Residue Rev.* 1969, **25**, 59–67.

Li, M. F. & C. Jordan: Use of spinner culture cells to detect water pollution. *J. Fish. Res. Bd. Canada* 1969, **26**, 1378–1382.

Mackenzie, C. G., J. B. Mackenzie & O. K. Reiss: In: *Lipid metabolism in tissue culture cells.* Ed.: G. H. Rothblat & D. Kritchevsky. The Wistar Symposium Monograph No 6. Wistar Inst. Press, Philadelphia, Pennsylvania 1967, pp. 63–81.

Mackenzie, J. B, C. G. Mackenzie & O. K. Reiss: Regulation of cell lipid metabolism and accumulation VII. Increase by glycerol of the polar lipid and triglyceride content of cultured cells. *Proc. Soc. Exptl. Biol. Med.* 1968, **128**, 42–46.

Moskowitz, M. S.: In: *Lipid metabolism in tissue culture cells.* Ed.: G. H. Rothblat & D. Kritchevsky. The Wistar Symposium Monograph No. 6. Wistar Inst. Press, Philadelphia, Pennsylvania 1967, pp. 49–59.

Mueller, G. C.: Biochemical events in the animal cell cycle. *Fed. Proc.* 1969, **28**, 1780–1789.

Sanford, K. K., W. R. Earle & G. D. Likely: The growth *in vitro* of single isolated tissue cells. *J. Nat. Cancer Inst.* 1948, **9**, 229–246.

Sykes, J. A. & E. B. Moore: A simple tissue chamber. *Texas Rep. Biol. Med.* 1960, **18**, 288–297.

Way, J. M.: Toxicity and hazards to man, domestic animals, and wildlife from some commonly used auxin herbicides. *Residue Rev.* 1969, **26**, 37–62.

Acta pharmacol. et toxicol. 1971, 29, 87–94.

From the Research Division of Pharmacia A/S,
Vanløse, Copenhagen, Denmark

The Hyperglycaemic Activity of Some Catecholamines and the Effect of α- and β-Adrenergic Blocking Compounds in the Mouse

By

Keld Hermansen and Inge Hyttel

(Received June 4, 1970)

Abstract: In order to classify the receptors involved in the hyperglycaemic response to catecholamines in the mouse, the relative potency of noradrenaline, adrenaline and isoprenaline in the induction of hyperglycaemia has been tested by giving increasing doses from 10 μg/kg to 1 mg/kg of one of the amines intraperitoneally. Following catecholamine administration the maximal concentration of glucose in the blood occurred between 10 and 30 min. and amounted to about 300 mg %. The relative potency was: adrenaline > noradrenaline while isoprenaline was inactive. The antagonistic effect on adrenaline induced hyperglycaemia of the α-adrenergic blocking compounds: phenoxybenzamine HCl (dibenzyline®) and phentolamine (regitin®) and the β-adrenergic blocking compounds: 1-(isopropylamino)-3-(o-phenoxyphenoxy)-2-propanol (Ph QA 33) and propranolol were tested by giving 1 and 10 mg/kg intraperitoneally 30 min. before the administration of adrenaline. Only phentolamine at the highest dose level was capable of preventing the hyperglycaemic response to adrenaline. Phenoxybenzamine, Ph QA 33 and propranolol proved to be inactive. It is concluded that the mechanism of adrenaline induced hyperglycaemia in mice cannot be explained simply by an α- and/or β-receptor activation.

Key-words: Catecholamines – hyperglycaemia – mice – sympatholytics.

The hyperglycaemic activity of different catecholamines and the antagonistic action of α- and β-adrenergic blocking compounds on this hyperglycaemia has been investigated extensively in several species including man (for survey see Himms-Hagen 1967). However, so far no investigations have been concerned with the type of receptors involved in the mechanism by which catecholamines induce metabolic changes in the mouse.

The present study was therefore undertaken in order to investigate whether hyperglycaemia induced by catecholamines in mice can be classified according to Ahlquist (1948) as an α- or β-receptor mediated effect. This was elucidated in two ways: (1) by determinating the hyperglycaemic potency

of noradrenaline, adrenaline and isoprenaline respectively, (2) and by testing the antagonistic action of α- and β-adrenergic blocking compounds on adrenaline induced hyperglycaemia.

Material and Methods

Test compounds.

The following catecholamines were used: adrenalini bitartras NFN; DL-N-Isopropylnoradrenaline hydrochloride (Fluka) = isoprenalini chloridum NFN and noradrenalini bitartras NFN; α-adrenergic blocking compounds: phenoxybenzamine HCl WHO (dibenzyline®) = benzylyti chloridum NFN and phentolaminum NFN (regitin®); β-adrenergic blocking compounds: 1-(isopropylamino)-3-(o-phenoxyphenoxy)-2-propanol, HCl (Ph QA 33) and propranolol (inderal®) = proprasylyti chloridum NFN. The doses of adrenaline, isoprenaline and noradrenaline are expressed as the base, phentolamine as the methanesulphonate; the other compounds are expressed in the form mentioned above. All the compounds were dissolved in saline in such a way that the volume given was 10 ml/kg body weight.

Effect of noradrenaline, adrenaline and isoprenaline on the blood sugar level.

Male unfasted NMRI mice weighing from 18–25 g were used. The mice were fed on Altromin® pellets and had free access to water. In order to minimize the influence of circadian variations in the blood sugar level all experiments were performed between 10 and 12 in the morning.

The hyperglycaemic potency of noradrenaline, adrenaline and isoprenaline was estimated in groups of five mice which were treated with increasing doses from 10 μg/kg to 1 mg/kg of one of the amines given intraperitoneally. 30 min. later the mice were sacrificed by decapitation. The blood from five mice in the same group was pooled in a heparinized centrifuge tube. The blood was then deproteinized by the addition of perchloric acid and the glucose concentration determined by means of a glucose oxidase-peroxidase method (Huggett & Nixon 1957).

In addition to the drug treated mice, each experiment included two groups of mice: which was left untreated and another which was treated with saline and served as control. All the doses were repeated four times on separate days so that the figures in table 1 represent the mean ± S. E. M. of four identical experiments except for the control groups, the figures of which represent the mean ± S. E. M. of eight experiments.

Antagonistic effect of α- and β-adrenergic blocking compounds.

The antagonistic action of α- and β-adrenergic blocking compounds on adrenaline induced hyperglycaemia was determined by giving 1 and 10 mg/kg of the compounds intraperitoneally into groups of five mice 30 min. before the administration of 0.1 mg/kg adrenaline by the same route. In these experiments too the mice were killed 30 min. after the administration of the amine

A possible effect of the antagonists *per se* on the blood sugar level as well as their adrenolytic effect was determined by running the following groups together: saline + saline, saline + adrenaline, antagonist + saline and antagonist + adrenaline. Each dose of antagonist was repeated four times together with the other groups, so that the columns in fig. 1 and 2 represent the mean of four determinations ± S. E. M

Results

Effect of noradrenaline, adrenaline and isoprenaline on the blood sugar level.

Table 1 shows the hyperglycaemic response to increasing doses of noradrenaline, adrenaline and isoprenaline estimated 30 min. after the administration of an amine. The figures demonstrate that treatment with saline only caused an insignificant increase in the blood sugar as compared to the untreated controls. Noradrenaline caused a dose-dependent increase. The lowest dose which raised the blood sugar level significantly as compared to the saline treated control was 100 μg/kg intraperitoneally. Adrenaline also increased the blood sugar in a dose-dependent fashion. 30 μg/kg intraperitoneally already had a significant effect. When the doses of adrenaline were raised above 0.3 mg/kg there was no further increase. When the hyperglycaemic action of noradrenaline and adrenaline are compared it is evident that adrenaline is more potent than noradrenaline at all dose levels. As seen from column 4 isoprenaline had no significant hyperglycaemic action.

Antagonistic action of α- and β-adrenergic blocking compounds on adrenaline induced hyperglycaemia.

It was shown above that adrenaline is by far the most potent amine in inducing hyperglycaemia in mice. 0.1 mg/kg adrenaline given intraperitoneally was therefore found suitable as a challenging dose for the experiments on the antagonistic action of α- and β-adrenergic blocking compounds.

Fig. 1 shows the blood sugar level after intraperitoneal administration of 1 and 10 mg/kg phenoxybenzamine and phentolamine respectively (light

Table 1.

Effect of noradrenaline, adrenaline and isoprenaline on the blood glucose level in mice.

Dose mg/kg i. p	Blood glucose mg %*		
	Noradrenaline	Adrenaline	Isoprenaline
No treatment	170 ± 7 (8)	170 ± 7 (8)	170 ± 7 (8)
Saline	184 ± 9 (8)	184 ± 9 (8)	184 ± 9 (8)
0.01	170 ± 4 (4)	210 ± 13ª (4)	179 ± 4 (4)
0 03	190 ± 7 (4)	233 ± 13ª (4)	190 ± 4 (4)
0.1	212 ± 5 (4)	285 ± 12ª (4)	191 ± 6 (6)
0.3	212 ± 18 (4)	328 ± 13ª (4)	166 ± 6 (4)
1.0	235 ± 3 (4)	311 ± 14ª (4)	160 ± 14 (4)

* Mean ± S. E. M. (n).

ª Significantly different from noradrenaline ($P < 0.05$).

columns). After the administration of 1 and 10 mg/kg of phenoxybenzamine, the blood sugar level increased from 155 ± 12 mg % in the saline treated control group to 178 ± 14 mg % and 178 ± 13 mg % ($\bar{x}$ ± S. E. M.) respectively. 1 mg/kg of phentolamine had no effect on the blood sugar, while 10 mg/kg phentolamine caused a decrease to 126 ± 9 mg %. None of the observed blood levels were, however, significantly different from the control level ($P > 0.05$).

Furthermore fig. 1 illustrates that administration of 0.1 mg/kg adrenaline caused an increase in blood sugar up to 262 ± 8 mg % in the saline pretreated group (hatched columns). Phenoxybenzamine had no antagonistic action on the hyperglycaemic action of adrenaline. Nor had 1 mg/kg phentolamine any blocking effect. On the contrary 10 mg/kg phentolamine completely abolished the hyperglycaemic response to adrenaline. As seen from fig. 1 the blood sugar level after intraperitoneal administration of 10 mg/kg phentolamine + adrenaline was 141 ± 15 mg % as compared to the adrenaline and phentolamine control values of 262 ± 8 mg % and 126 ± 9 mg % respectively.

Fig. 2 shows that neither 1 and 10 mg/kg of the β-adrenergic blocking compound Ph QA 33 nor the low dose of propranolol had any effect on the

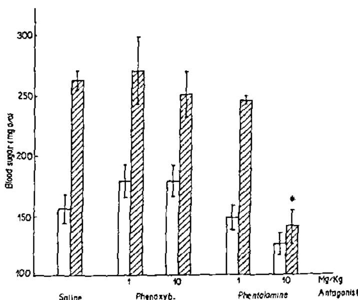


Fig. 1. Effect of phenoxybenzamine and phentolamine on adrenaline induced hyperglycaemia. The light columns depict the blood sugar level after intraperitoneal administration of antagonist + saline, and the hatched columns after antagonist + adrenaline (0.1 mg/kg intraperitoneally). The vertical lines indicate the S. E. M. (n = 4), the asterisk that the result differs significantly from the control value ($P < 0.05$).

blood sugar level as compared with the saline treated control group. 10 mg/kg propranolol caused an increase to 207 ± 18 mg % (light columns). This level was however not significantly different from that of the saline treated control group (179 ± 13 mg %).

Fig. 2 also demonstrates that none of the tested doses of Ph QA 33 and propranolol had any antagonistic action on adrenaline induced hyperglycaemia (hatched columns).

Discussion

The hyperglycaemic action of catecholamines appears to be a resultant of several independent effects. As such an activation of the adenylcyclase system leading to an increase in the intracellular concentration of 3',5'-AMP may be mentioned. This in turn accelerates the phosphorylase activity of the liver and thereby the breakdown of glycogen which is probably the major source of blood glucose.

Studies on the relative potency of noradrenaline, adrenaline and isoprenaline in inducing a certain pharmacological effect has been used in many

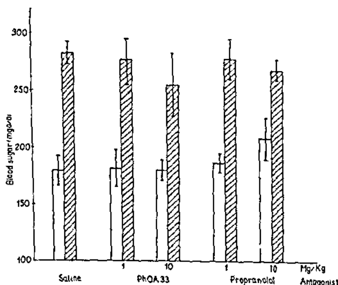


Fig. 2. Effect of Ph QA 33 and propranolol on adrenaline induced hyperglycaemia. The light columns depict the blood sugar level after intraperitoneal administration of antagonist + saline, and the hatched columns after antagonist + adrenaline (0.1 mg/kg intraperitoneally). The vertical lines indicate the S. E. M. (n = 4).

investigations in order to characterize a response as mediated via α- or β-adrenergic receptors.

In preliminary experiments the time course of the blood sugar changes induced by adrenaline and noradrenaline showed a similar pattern reaching a maximum between 20 and 30 min. after drug administration. Isoprenaline proved to be inactive. It therefore seemed justifiable to choose the blood sugar determination 30 min. after administration for comparing the hyperglycaemic potency of the three catecholamines (table 1).

In the present investigation the rank order as to hyperglycaemic potency was found to be adrenaline > noradrenaline, while isoprenaline was inactive. This indicates that hyperglycaemia in mice is mediated via α-receptors as has also been suggested in man (HUNNINGHAKE *et al.* 1966; PORTE 1967). In fasted rats and dogs the order of potency is adrenaline > isoprenaline ≫ noradrenaline, typical of a β-receptor stimulation (FLEMING & KENNY 1964; KVAM *et al.* 1965). The experiments with α- and β-blocking compounds support the view that adrenaline induced hyperglycaemia in mice is unrelated to β-receptor activation. Thus neither Ph QA 33 nor propranolol in doses considerably above those causing a significant blockade of cardiac β-receptors in the same species, had any antagonistic action on the adrenaline-induced hyperglycaemia (HERMANSEN 1968 & 1970). Propanolol by itself in a dose of 10 mg/kg intraperitoneally revealed a slight though not significant increase in the blood glucose level. This is in accordance with previous findings by ESTLER & AMMON (1966) who suggest that the hyperglycaemic effect of propranolol in mice may be due to a reduction of the energy requirements and carbohydrate metabolism as a consequence of the blockade of cardiac β-receptors.

The α-adrenergic blocking compounds phenoxybenzamine and phentolamine were used in doses of 1 and 10 mg/kg intraperitoneally into the mice. These doses cause a significant increase in the skin temperature which is probably an indication of α-adrenergic blocking potency (own observations; RICHTER 1964).

Phentolamine was the only compound which significantly antagonized the hyperglycaemic action of adrenaline. This agent by itself caused a small though insignificant lowering of the blood sugar level when 10 mg/kg was administered intraperitoneally. A hypoglycaemic effect of phentolamine has also been observed in rats, dogs and human subjects (BOSHART *et al.* 1965; SHERIF *et al.* 1957). The fact that phentolamine completely abolished the adrenaline hyperglycaemia indicates that α-adrenergic receptors may be involved in the development of the hyperglycaemic response. Against this view, however, is the observation that another α-adrenergic blocking compound phenoxybenzamine had no inhibiting effect on the action of adrenaline.

The observed differences in the antagonistic action on the adrenaline induced hyperglycaemia might be due to differences in the onset and duration of the α- or β-blocking effect induced by the compounds. However, adrenaline was administered 30 min. after the drugs in all experiments. At this time their blocking action is evident on other parameters. It therefore seems unlikely that the differences in pharmacokinetics should be responsible for the observed differences.

From these experiments it may thus be concluded that catecholamine induced hyperglycaemia in mice is mediated via mechanisms susceptible to phentolamine but not to phenoxybenzamine or β-adrenergic blocking compounds. This action of catecholamines in mice can therefore not be classified according to AHLQUIST (1948) as a classical α- or β-receptor mediated effect.

Acknowledgements

The authors wish to thank Imperial Chemical Industries Ltd., Macclesfield for a generous supply of propranolol.

REFERENCES

Ahlquist, R. P.: A study of the adrenotropic receptors. *Amer. J. Physiol.* 1948, **153**, 586–600.

Boshart, C. R., L. Will, A. Pirré & I. Ringler: The effects of reserpine, guanethidine and other autonomic drugs on free fatty acid mobilization induced by phentolamine. *J. Pharmacol. exp. Therap.* 1965, **149**, 57–64.

Estler, C. J. & H. P. T. Ammon: The influence of the β-sympathicolytic agent propranolol on glycogenolysis and glycolysis in muscle, brain and liver of white mice. *Biochem. Pharmacol.* 1966, **15**, 2031–2035.

Fleming, W. W. & A. D Kenny: The effect of fasting on the hyperglycaemic responses to catechol amines in rats. *Brit. J. Pharmacol.* 1964, **22**, 267–274.

Hermansen, K.: Some pharmacological properties of a new β-adrenergic blocking agent, 1-(isopropylamino)-3-(o-phenoxyphenoxy)-2-propanol, HCl (Ph QA 33). *Acta pharmacol. et toxicol.* 1968, **26**, 343–362.

Hermansen, K.: Antifibrillatory effect of some β-adrenergic receptor blocking agents determined by a new test procedure in mice. *Acta pharmacol. et toxicol.* 1970, **28**, 17–27.

Himms-Hagen, J.: Sympathetic regulation of metabolism. *Pharmacol. Rev.* 1967, **19**, 367–461.

Huggett, A. St. G. & D. A. Nixon: Use of glucose oxidase, peroxidase and o-dianisidine in determination of blood and urinary glucose. *Lancet* 1957, **273**, 368–370.

Hunninghake, D. B., D. L. Azarnoff & D. Waxman: The effect of butoxamine on catecholamine-induced metabolic changes in humans. *Clin. Pharmacol. Therap.* 1966, **7**, 470–476.

Kvam, D. C., D. A. Riggilo & P. M. Lish: Effect of some new β-adrenergic blocking agents on certain metabolic responses to catecholamines. *J. Pharmacol. exp. Therap.* 1965, **149**, 183–192.

Porte, D. Jr.: A receptor mechanism for the inhibition of insulin release by epinephrine in man. *J. Clin. Invest.* 1967, **46**, 86–94.
Richter, W.: Estimation of vasodilator drug effects in mice by measurements of paw skin temperature. *Acta pharmacol. et toxicol.* 1964, **21**, 91–104.
Sherif, M. A. F., M. A. Razzak & A. M. Hassaballak: Comparative study of some imidazoline derivatives and dihydrogenated ergot alkaloids on blood sugar and gastric acidity. *Arch. int. Pharmacodyn.* 1957, **110**, 1–9.

Acta pharmacol. et toxicol. 1971, 29, 95–105.

From the Research Laboratories of the State Alcohol
Monopoly (Alko), Helsinki, Finland

Thyroid State and Voluntary Alcohol Consumption of Albino Rats

By

M. E. Hillbom

(Received June 22, 1970)

Abstract: Male Wistar rats were made hypo- and hyperthyroid in a period of three weeks by the daily administration of propylthiouracil or 3,3',5-triiodo-L-thyronine, and the voluntary alcohol consumption of the animals was investigated. The triiodothyronine treatment increased, and propylthiouracil treatment decreased the total caloric intake of the animals. However, the portion of ethanol in the total caloric intake of the rats was significantly increased by propylthiouracil treatment and significantly decreased by triiodothyronine treatment while the total caloric intake did not correlate positively with the voluntary alcohol consumption of these animals. The absorption of ethanol from the intravascular space, the peritoneal cavity and the gastrointestinal tract as well as the rate of elimination of ethanol were increased by triiodothyronine treatment and decreased by propylthiouracil treatment. The acetaldehyde concentration during oxidation of ethanol was found to be higher (166 ± 22 nmol/ml of blood) in the hepatic venous blood of euthyroid animals, as compared with hypo- and hyperthyroid ones. Acetaldehyde accumulation was not found to be a factor regulating voluntary alcohol intake in these animals.

Key-words: Ethanol intake – thyroid state – acetaldehyde.

In 1953, ZARROW & ROSENBERG reported that propylthiouracil mixed into commercial laboratory food increased the voluntary alcohol intake of the rat. They came to the conclusion that the action of the goitrogen was independent of the thyroid and could not be prevented by giving thyroxine or simulated by thyroidectomy. However, RICHTER (1956) was later able to demonstrate that thyroid powder mixed in food decreased the voluntary alcohol intake of albino rats. Subsequently, however, PRIETO *et al.* (1958) were unable to confirm his finding and thyroidectomy was claimed either to increase (LEMAGNEN 1960; RICHTER 1957) or decrease (ASCHKENASY-LELU 1962) alcohol intake. Iodine deficiency has been reported to decrease alcohol consumption in mice (MIRONE 1957) but to increase it in rats (MÄENPÄÄ & FORSANDER 1966).

Recently, MURDOCK (1969) reported that triiodothyronine (liothyroninum NFN) injections (75–100 μg/day) sharply reduced the ethanol consumption of rats during the period in which they were in the hyperthyroid state. These partially conflicting data inspired a study of some basic effects of ethanol in rats pretreated with propylthiouracil or triiodothyronine in relation to voluntary alcohol intake.

As stressed by FORSANDER (1967), ethanol has two fundamentally different effects in the organism. On the one hand it has a pharmacological effect, reflected in intoxication, and on the other hand, a metabolic effect, due to its caloric value. The pharmacological effect of ethanol in regulating voluntary ethanol intake was approached by determining the absorption of ethanol administered intraperitoneally and via the gastrointestinal tract and also by determining the concentration of acetaldehyde formed in the liver during elimination of various amounts of ethanol. Acetaldehyde has been recognized as directly responsible for a large number of the features of the acute and chronic intoxication syndromes produced by ethanol (TRUITT & DURITZ 1967). The *in vivo* rate of elimination of ethanol and the contribution of ethanol to the total intake of exogenous calories were determined in hypo-, hyper- and euthyroid animals in order to obtain some information about the metabolic actions of ethanol.

Material and Methods

Male Wistar rats, four months old, which had previously been kept for one month on a free choice between tap water and 10 % ethanol (v/v) and given ordinary laboratory food *ad libitum*, were used for the experiments. During the procedure all the rats were kept in individual cages and again given a choice of water and 10 % ethanol (v/v). The position of the drinking bottles was changed every three days in order to avoid habituation. Ordinary laboratory food was given *ad libitum* and the daily consumption of food and ethanol was recorded. The details of the method have been given by ERIKSSON (1969). In this study, alcohol consumption was not measured as the relative alcohol preference but as the consumption of absolute alcohol, since the latter has been shown to be more significant for the interpretation of the results in a study of this kind (ERIKSSON 1967) After two weeks, some of the animals were given daily doses of propylthiouracil (PTU, obtained from Eli Lilly and Co., Indianapolis, USA), 5 mg per 100 g body weight of a 0.5 % solution administered by stomach tube, while others received 3,3′,5-triiodo-L-thyronine (T_3, Sigma Chemical Co., Missouri, USA) in daily doses of 20 μg per 100 g body weight intraperitoneally in saline. The special treatments were continued for five weeks. Control animals were given saline. The oxygen consumption of the experimental animals in basal conditions was recorded at the end of the third week by means of a Beckman Oxygen Analyser, Model E 2; the method used was that described by DEPOCAS & HART (1957).

A similarly treated group of rats which had not been given ethanol was used for other experiments. The elimination rate of ethanol was determined by giving a single intraperitoneal injection of ethanol, 1.6 ml per 100 g body weight, in saline as a 2 M

solution. Blood samples were taken from the tip of the tail in order to determine the elimination time of the ethanol, which was obtained by extrapolating the linear descending limb of the blood alcohol curve to the abscissa. The amount of ethanol injected was then divided by the elimination time in order to arrive at the elimination rate. The same amount of ethanol was injected when studying the absorption of ethanol from the peritoneal cavity.

The absorption of ethanol from the gastrointestinal tract was determined by following the blood ethanol concentration after a single oral dose of 2 M ethanol of 1.0 ml per 100 g body weight. Ethanol, 0.5 ml per 100 g body weight, was also injected intravenously into the femoral vein as a 2 M solution dissolved in saline, and the blood alcohol concentration was determined after five minutes.

Finally the influence of various amounts of ethanol on the liberation of acetaldehyde in the liver during ethanol metabolism was investigated in hypo-, hyper- and euthyroid rats. The animals were anaesthetised with 40 mg of pentobarbital (mebumalum NFN) per kg of body weight and 15 minutes later 0.2 ml of 2 M ethanol per 100 g body weight in saline was injected into the femoral vein. The abdomen was then opened and 5 minutes after the injection 0.5 ml of blood was withdrawn from the hepatic vein. Immediately afterwards, 0.6 ml of 2 M ethanol per 100 g body weight was injected in saline into the femoral vein. Three other samples of blood, each of 0.5 ml were then drawn from the hepatic vein.

Ethanol and acetaldehyde were measured in the acid sample by gas chromatography. The head-space technique was applied with a Perkin-Elmer F 40 gas chromatograph. The temperature of the water bath used for the production of the gas space was 65°. Tert-butanol was used as the internal standard. No corrections were made for acetaldehyde formation from pyruvate or from ethanol during the preparation of samples, since control experiments showed that only minor amounts were formed in an acid solution.

Results

The experimental animals attained marked hypo- and hyperthyroid states within three weeks of treatment (table 1), as shown by significant ($P < 0.001$) differences in basal oxygen consumption between the PTU-treated, control

Table 1.

Basal oxygen consumption and rate of elimination of ethanol of the experimental animals.

Group	Number of animals	Basal oxygen consumption (ml/hr/100 g)	Rate of elimination of ethanol (ml/hr/100 g)
PTU-treated	9	0.42 ± 0.04	33.8 ± 11.8
Control	9	0.61 ± 0.08	40.0 ± 6.8
T_3-treated	9	0.84 ± 0.09	44.3 ± 8.4

The figures represent means ± S. D.

Table 2.

Changes with time in voluntary alcohol consumption and total caloric intake.

Time (days)	Voluntary alcohol consumption (g of abs. alc./100 g body wt./day)		Total caloric intake (cal./100 g body wt./day)	
	PTU-treated	Controls	PTU-treated	Controls
12*	0.17 ± 0.09	0.18 ± 0.12	17.2 ± 0.7	17.3 ± 1.3
24	0.26 ± 0.11	0.21 ± 0.11	15.3 ± 0.7**	17.1 ± 1.5
36	0.39 ± 0.14**	0.17 ± 0.15	13.9 ± 1.2**	17.7 ± 2.1
48	0.34 ± 0.16**	0.17 ± 0.11	13.6 ± 1.0**	17.5 ± 1.0
	T_3-treated	Controls	T_3-treated	Controls
12*	0.39 ± 0.18	0.34 ± 0.12	18.4 ± 1.2	18.8 ± 0.6
24	0.38 ± 0.13**	0.53 ± 0.07	23.0 ± 1.2**	18.2 ± 1.0
36	0.43 ± 0.19	0.53 ± 0.08	32.9 ± 2.2**	18.3 ± 1.1
48	0.38 ± 0.21	0.47 ± 0.12	31.6 ± 1.4**	17.9 ± 1.1

* Control period without treatment for twelve days.

** $P < 0.01$ for difference between treated and corresponding control rats. The figures represent the mean value of 9 animals ± S D.

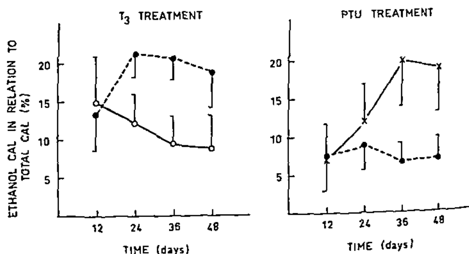


Fig. 1. Influence of T_3 and *PTU* treatment on *the ratio of ethanol calories* to total exogenous calories during a 48-day experiment. The caloric contribution of the voluntarily consumed ethanol was decreased by T_3 treatment (O——O) *and increased by PTU treatment* (X——X). The dotted lines (●- - - -●) represent the values of the control groups. Each point in the figure represents the mean value of 9 animals ± *S. D.*

and T_3-treated groups. Within the same time the effects of the drugs on voluntary alcohol consumption and on total caloric intake also proved to be maximal (table 2). PTU increased the consumption of absolute alcohol during the first twelve days of treatment (table 2). As compared with the control animals, this effect became statistically significant ($P < 0.01$) within three weeks and could better be seen when the caloric portion of the voluntarily consumed ethanol was compared with the total amount of exogenous calories ingested by the rat (fig. 1). T_3 treatment did not produce any marked change in the amount of absolute alcohol consumed, but since the total caloric intake of these rats was nearly doubled during the treatment, the caloric contribution of the ethanol was significantly ($P < 0.05$) reduced (fig. 1).

In contrast to the effects of the drugs on voluntary alcohol consumption, PTU treatment decreased and T_3 treatment increased the *in vivo* rate of elimination of ethanol (table 1). However, these effects were not statistically significant ($P > 0.05$). In these animals the *in vivo* rate of elimination of ethanol did not correspond to the ethanol-oxidizing capacity of liver tissue, since the latter parameter was found to be decreased in both hypo- and hyperthyroid rats (HILLBOM & PIKKARAINEN 1970). The increased *in vivo* rate of elimination of ethanol in hyperthyroid rats can be explained by the increased liver-to-body weight ratio of these animals (HILLBOM 1970).

The blood alcohol curves (fig. 2) after intraperitoneal administration revealed that the absorption of ethanol from the peritoneal cavity was markedly depressed in hypothyroid and increased in hyperthyroid animals. Ten minutes after the injection the blood alcohol concentration was signifi-

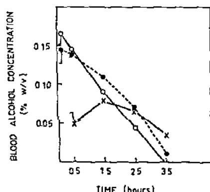


Fig. 2. Blood alcohol curves of T_3- (O——O), PTU-treated (X——X) and control rats (●- - - -●). Ethanol in saline was injected intraperitoneally 1.6 ml per 100 g body weight as a 2 M solution. Each point in the figure represents the mean ± S.D. of 9 animals.

Table 3.

Blood alcohol concentrations after a single oral or intravenous dose of 2 M ethanol.

Group	Number of animals	Blood ethanol concentration (%)	
Ethanol i. v. (0.5 ml/100 g)		5 min. after injection	
PTU-treated	8	0.082 ± 0.010	
Control	8	0.073 ± 0.010	
T_3-treated	8	0.058 ± 0.005	
Ethanol per os (1.0 ml/100 g)			*30 min. after injection*
PTU-treated	9	0.014 ± 0.006	0.044 ± 0.016
Control	9	0.024 ± 0.014	0.078 ± 0.010
T_3-treated	9	0.041 ± 0.013	0.075 ± 0.024

The figures represent means ± S. D.

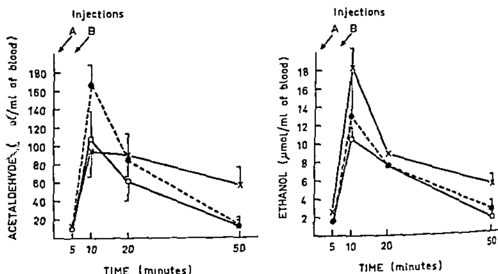


Fig. 3. Concentration of acetaldehyde and ethanol in hepatic venous blood of control (●- - - -●), *PTU-* (×——×) *and T_3-treated* (○——○) rats. A small amount of 2 M ethanol (0.2 ml/100 g body weight) in saline was first injected into the femoral vein (injection A). Five min. later a sample was *taken from hepatic* venous blood and a second injection of ethanol (injection B; 0.6 ml/100 g body weight of 2 M ethanol in saline) was given into the femoral vein. Each point in *the figure represents* the mean ± S. D. of 8 animals.

cantly ($P < 0.05$) higher in the hyperthyroid than in the control rats. The elimination curve of ethanol in hypothyroid rats did not become rectilinear during the elimination time and the maximal blood alcohol concentration reached only about half the values found in hyper- and euthyroid animals.

Five minutes after a single intravenous injection of ethanol the blood alcohol concentration was significantly ($P < 0.05$) higher in PTU-treated and lower ($P < 0.005$) in T_3-treated rats than in the controls (table 3). When ethanol was given orally, the blood alcohol concentration five minutes after administration was significantly ($P < 0.05$) higher in T_3-treated rats than in the other two groups and 30 minutes after ethanol administration it was significantly ($P < 0.001$) lower in the PTU-treated rats. These results proved that absorption of ethanol from the intravascular space and the gastrointestinal tract was increased in hyperthyroid and decreased in hypothyroid animals.

No marked differences in the acetaldehyde concentration of the blood were found between the experimental groups when small amounts of ethanol (< 0.02 %) were metabolized (fig. 3, initial points). An increase in blood ethanol concentration was followed by an increase in blood acetaldehyde concentration in all the rats. However, in hypothyroid rats the blood acetaldehyde level did not exceed 100 nmoles per ml of blood, despite the fact that the highest ethanol concentration was found in the blood of these animals. In the euthyroid group the acetaldehyde level was found to be significantly ($P < 0.001$) higher than in the other two groups.

Discussion

Richter (1941) has suggested that alcohol preference in rats is based on their ability to utilize alcohol as a source of calories. The validity of this idea has subsequently been widely investigated and some of the results have been criticized by Lester (1966) in his review on the aetiology of alcoholism. Quite recently, in free choice experiments with rats, a marked negative correlation has been demonstrated between the calories obtained from food and those obtained from alcohol (Eriksson 1969). On the basis of experiments with mice, it has also been reported that the proportion of ethanol-derived calories, rather than the absolute concentration of ethanol in liquid diets, appears to limit the animal's capacity to consume alcohol (Freund 1970).

In the present study, the total caloric intake of rats was modified by PTU or T_3 treatment. Assuming that ethanol-derived calories regulate the voluntary alcohol consumption of the albino rat, it was to be anticipated that hyperthyroid animals, with their increased total caloric intake, would increase their

alcohol consumption and that the hypothyroid animals would behave in the opposite way. However, this was not found to be the case and the results showed convincingly that voluntary alcohol consumption is not positively correlated with total caloric intake. Since the capacity of liver tissue to oxidize ethanol is almost the same in hypo- and hyperthyroid rats and somewhat decreased in both as compared to euthyroid animals (HILLBOM & PIKKARAINEN 1970), it seems very unlikely that the proportion of ethanol-derived calories is the sole factor concerned in regulating the voluntary alcohol consumption of these animals. Other possibilities must be taken into account.

Since the discovery of the effect of disulfiram (HALD & JACOBSEN 1948) on alcohol consumption, ethanol hypersensitivity caused by various chemically different drugs has been studied in relation to acetaldehyde accumulation (WAGNER 1957). It has been claimed that the potent metabolic action of acetaldehyde occurs at concentrations sufficient to explain many of the toxic depressant effects of heavy and prolonged drinking on the brain and other tissues (TRUITT & DURITZ 1967). In the brain for instance, a 50 % inactivation of coenzyme A is brought about by a concentration of 200 nmoles of acetaldehyde per ml of blood (AMMON *et al.* 1969). The sulphonylurea-induced "antabuse" syndrome has been thought to be due either to an increase in acetaldehyde levels or to an alteration in the metabolism of 5-hydroxytryptamine (PODGAINY & BRESSLER 1968). However, conflicting results were found concerning an ethanol-induced shift in the metabolism of 5-hydroxytryptamine in the brain (DAVIS *et al.* 1967; TYCE *et al.* 1968) and no correlation was found between the blood levels of acetaldehyde and the "antabuse" syndrome in diabetic patients on sulphonylureas after ethanol ingestion (FITZGERALD *et al.* 1962). On the other hand, it must be remembered that acetaldehyde levels are much lower in peripheral blood than in hepatic venous blood (FORSANDER *et al.* 1969). The levels in the arterial blood of the brain however, have not been measured.

In the present study it was found that there was no correlation between acetaldehyde levels in hepatic venous blood and the voluntary alcohol consumption of the experimental groups, since no accumulation of acetaldehyde occurred in the hyperthyroid rats. Many compounds, in combination with alcohol, are known to produce unpleasant effects without influencing acetaldehyde metabolism (BÜTTNER 1960). Butyraldoxime, for instance, does not bring about an accumulation of acetaldehyde in the rat (FORSANDER 1970). Moreover, some nutritional states which produce sobriety in rats are not connected with acetaldehyde accumulation during ethanol metabolism. It was previously reported that rats given a low-protein, high-fat diet, deficient in choline chloride avoid drinking ethyl alcohol (HILLBOM 1967), and preliminary experiments in this laboratory (HILLBOM, unpublished observation) have revealed that in these rats too there is no accumulation of acetaldehyde. So

other factors must account for the ethanol hypersensitivity in these animals.

Since the absorption of ethanol given *per os* was increased after T_3 treatment and decreased after PTU treatment, it can be argued that the deleterious effects of ethanol which produce the intoxication syndrome are more rapidly developed in hyperthyroid rats and delayed in hypothyroid ones. On the other hand, it has been reported that enormous doses of triiodothyronine given before alcohol intake lead to a small but significant diminution in the degree of drunkenness in dogs (NEWMAN & SMITH 1959) and that the rate of elimination of ethanol is not altered in triiodothyroninetreated dogs (NEWMAN & SMITH 1959; SMITH *et al.* 1963).

RICHTER (1956) reported that forced feeding of hyperthyroid rats with alcohol produced demonstrable lesions in the adrenals and kidneys. The pathogenesis of these lesions has not been resolved. Some metabolic features have been found, however, which may contribute to the ethanol hypersensitivity of hyperthyroid rats. Ethanol produce significant hypoglycaemia in hyperthyroid patients (more severe than in controls), in whom hepatic glycogen stores are known to be depleted (ARKY & FREINKEL 1966). In hyperthyroid rats ethanol has been found to produce severe hypoglycaemia (YLIKAHRI 1969) and moderate ketosis (LINDROS 1970). Hypothyroid rats, on the other hand, do not develop ketosis after ethanol administration (Ibid). The course of the development of ketosis in connexion with voluntary alcohol consumption in the albino rat was reported by FORSANDER & SALASPURO (1962). They found a marked negative correlation between ethanol preference and the degree of ketone body excretion in the urine after butyrate administration. On the basis of the evidence presented here, it cannot be ruled out that the ethanol-induced development of ketosis or hypoglycaemia or both together are responsible for the differences in the capacity to consume ethanol observed in hypo-, hyper- and euthyroid rats.

REFERENCES

Ammon, H. P. T., C.-J. Estler & F. Heim: Inactivation of coenzyme A by ethanol. Acetaldehyde as mediator of the inactivation of coenzyme A following the administration of ethanol *in vivo*. *Biochem. Pharmacol.* 1969, 18, 29–33.

Arky, R. A. & N. Freinkel: Alcohol hypoglycemia. V. Alcohol infusion to test gluconeogenesis in starvation with special reference to obesity. *New Engl J. Med.* 1966, 274, 420–433.

Aschkenasy-Lelu, P.: L'action inhibitrice des oestrogènes sur la consommation élective d'alcool du rat passe-t-elle par un relais thyroïdien? *Arch. Sci. Physiol.* 1962, 16, 203–211

Büttner, H : Arzneimittel und Alkohol. *Ther. Gegenw.* 1960, 99, 384–386.

Davis, V. E., H. Brown, J. A. Huff & J. L. Cashaw: The alteration of serotonin metabolism to 5-hydroxytryptophol by ethanol ingestion in man. *J. Lab clin. Med.* 1967, 69, 132–140.

Depocas, F. & J. S. Hart: Use of the Pauling oxygen analyzer for measurement of oxygen consumption of animals in open-circuit systems and in a short-lag, closed-circuit apparatus. *J. appl. Physiol.* 1957, 10, 388–392.

Eriksson, K.: Effect of two diuretic drugs on liquid consumption and free choice of alcohol in albino rats. *Nature* (Lond.) 1967, **213**, 316–317.

Eriksson, K.: Factors affecting voluntary alcohol consumption in the albino rat. *Ann. Zool. Fennici* 1969, **6**, 227–265.

Fitzgerald, M. G., R. Gaddie, J. M. Malins & D. J. O'Sullivan: Alcohol sensitivity in diabetics receiving chlorpropamide. *Diabetes* 1962, **11**, 40–43.

Forsander, O. A.: The role of metabolism in alcohol consumption. In: *Biochemical factors in alcoholism*. Ed. by R. P. Maickel. Pergamon Press, Oxford 1967, pp. 7–16.

Forsander, O. A.: Influence of ethanol and butyraldoxime on liver metabolism. *Biochem. Pharmacol.* 1970, **19**, 2131–2136.

Forsander, O. A. & M. P. Salaspuro: Voluntary ethanol consumption as related to ketone bodies metabolism in rats. *Life Sci.* 1962, **9**, 467–470.

Forsander, O. A., M. E. Hillbom & K. O. Lindros: Influence of thyroid function on the acetaldehyde level of blood and liver of intact rats during ethanol metabolism. *Acta pharmacol. et toxicol.* 1969, **27**, 410–416.

Freund, G.: Alcohol consumption and its circadian distribution in mice. *J. Nutr.* 1970, **100**, 30–36.

Hald, J. & E. Jacobsen: Formation of acetaldehyde in the organism after ingestion of antabuse. *Acta pharmacol. et toxicol.* 1948, **4**, 305–310.

Hillbom, M. E.: The effect of choline chloride on the voluntary alcohol consumption of the albino rat. *Japan. J. Stud. Alc.* 1967, **2**, 111–114.

Hillbom, M. E.: Inhibition of sorbitol oxidation by ethanol in intact rats pretreated with triiodothyronine or propyl thiouracil. *Biochem. Pharmacol.* 1970, **19**, 483–486.

Hillbom, M. E. & P. H. Pikkarainen: Liver alcohol and sorbitol dehydrogenase activities in hypo- and hyperthyroid rats. *Biochem. Pharmacol.* 1970, **19**, 2097–2103.

LeMagnen, J.: Etude de quelques facteurs associés à des modifications de la consommation spontanée d'alcool éthylique par le rat. *J. Physiol.* (Paris) 1960, **52**, 873–884.

Lester, D.: Self-selection of alcohol by animals, human variation and the etiology of alcoholism. A critical review. *Quart. J. Stud. Alc.* 1966, **27**, 395–438.

Lindros, K. O: Interference of ethanol and sorbitol with hepatic ketone body metabolism in normal, hyper- and hypothyroid rats *Eur. J. Biochem* 1970, **13**, 111–116.

Mirone, L.: Dietary deficiency in mice in relation to voluntary alcohol consumption. *Quart. J. Stud. Alc.* 1957, **18**, 552–560.

Murdock, H. R., Jr.: Relationship between ethanol metabolism and thyroid function. In: *Biochemical and clinical aspects of alcohol metabolism*. Ed by V. M. Sardesai. Charles C. Thomas, Springfield, Illinois 1969, pp. 235–240.

Mäenpää, P. H. & O. A. Forsander: Influence of iodine deficiency on free choice between alcohol and water in rats. *Quart. J. Stud. Alc.* 1966, **27**, 596–603.

Newman, H. & M. E. Smith: Triiodothyronine in acute alcoholic intoxication. *Nature* (Lond.) 1959, **183**, 689–690.

Podgainy, H. & R. Bressler: Biochemical basis of the sulfonylureainduced antabuse syndrome. *Diabetes* 1968, **17**, 679–683.

Prieto, R., A. Varela & J. Mardones: Influence of oral administration of thyroid powder on the voluntary alcohol intake by rats. *Acta physiol. latinoam.* 1958, **8**, 203.

Richter, C. P.: Alcohol as food. *Quart. J. Stud. Alc.* 1941, **1**, 650–662.

Richter, C. P.: Loss of appetite for alcohol and alcoholic beverages produced in rats by treatment with thyroid preparations. *Endocrinology* 1956, **59**, 472–478.

Richter, C. P.: Loss of appetite for alcohol and alcoholic beverages produced in rats by treatment with thyroid preparations. In: *Alcoholism, basic aspects and treatment.* Ed. by H. E. Himwich. American Association for the Advancement of Science, Washington, D. C. 1957, pp. 59–65.

Smith, D. E., N. E. Fallis & L. Tetreault: Effect of triiodothyronine on rate of alcohol oxidation in dogs *New Engl. J. Med.* 1963, **268**, 91–92.

Truitt, E. B., Jr. & G. Duritz: The role of acetaldehyde in the actions of ethanol. In: *Biochemical factors in alcoholism.* Ed. by R. P. Maickel, Pergamon Press, Oxford 1967, pp. 61–69.

Tyce, G. M., E. V. Flock & C. A. Owen: 5-hydroxytryptamine metabolism in brains of ethanol-intoxicated rats. *Mayo Clin. Proc.* 1968, **43**, 668–673.

Wagner, H.-J.: Einfluss von Medikamenten auf den Acetaldehydspiegel im Blut nach Alkoholzufuhr (entzymatische Bestimmung des Acetaldehyd). *Deut. Z. ges. gerichtl. Med.* 1957, **46**, 70–78.

Ylikahri, R.: Ethanol hypoglycemia in thyroxine-treated rats. *Scand. J. clin. Lab. Invest.* 1969, **23**, Suppl. 108, 58.

Zarrow, M. X. & B. Rosenberg· Alcoholic drive in rats treated with propyl thiouracil. *Am. J. Physiol.* 1953, **172**, 141–146.

From the Department of Pharmacology, University of Bergen.
Bergen, Norway

Minor Urinary Metabolites of L-Dopa in the Rat

By

Olav M. Bakke

(Received September 3, 1970)

Key-words: L-Dopa – *rats* – *metabolism.*

Biotransformation of L-dopa by intestinal microorganisms was suggested by SANDLER *et al.* (1969) who found that the quantity of *meta*-hydroxyphenylacetic acid in the urine of patients with parkinsonism treated with large doses of L-dopa decreased significantly when neomycin was given orally. The present report describes some simple phenolic L-dopa metabolites which are dependent on a normally functioning intestinal microflora.

Male albino rats aged 5–6 months and weighing 303–335 g were used. The animals were fed a commercial pellet diet (Felleskjøpet, Oslo) before the experiments. Then, 1 % w/w L-dopa (eldopar®, Weiders Farmasøytiske A/S, Oslo) was added to a purified diet described previously (BAKKE 1969a) and fed *ad libitum.* The mean 24 hour intake of L-dopa during the 4 day experimental period ranged from 120 to 180 mg. A second experimental group received the L-dopa diet with 1 % w/w neomycin sulphate while the control rats received only the purified diet.

Urines were collected at $-20°$ from individual metabolic cages for 48 hours starting 2 days after changing to the experimental diets. They were thawed, filtered and diluted to 20 ml with distilled water. Aliquots (10 ml) of the diluted urines were hydrolyzed with β-glucuronidase and sulphatase and analyzed by gas chromatography (GLC) on 3 columns and by thin-layer chromatography (TLC) in 2 solvent systems as described previously (BAKKE 1969a).

The urines from 4 of 5 rats given L-dopa without neomycin sulphate contained 4-methylcatechol in amounts ranging from trace (TLC) to 1.2 mg/24 hours (GLC). The animals which excreted this compound also produced 4-methylguaiacol which was detected in the urine by gas chromatography only (trace-0.04 mg/24 hours). The chromatograms from a 2 % w/w tricresylphosphate column of extracts from all 5 urines in this group showed peaks with the retention characteristics of *meta*-cresol (trace-approximately 0.4 mg/24 hours) and positive spiking tests. Further identification and exact quantita-

tion of this metabolite were not possible because *meta*-cresol coincides with the naturally occurring *para*-cresol on the other 2 columns used. The present study with unlabelled L-dopa does not exclude *para*-cresol as an additional minor metabolite. The control animals given purified diet alone or the diet to which L-dopa plus neomycin had been added did not excrete 4-methylcatechol, 4-methylguaiacol or *meta*-cresol.

The phenolic acid metabolites of L-dopa in rat urine were not investigated in detail. However, the thin-layer chromatograms contained spots corresponding to 3,4-dihydroxyphenylacetic acid, *meta*-hydroxyphenylacetic acid, homovanillic acid and homoisovanillic acid. These compounds are also known to be metabolites of L-dopa in man (O'GORMAN *et al.* 1970).

The intestinal microflora carries out numerous metabolic reactions including the formation of phenolic acids from L-tyrosine (BAKKE 1969b), decarboxylation of phenolic acids with free hydroxyl groups in the *para* position and removal of such hydroxyl groups (SCHELINE 1968). The failure to detect 4-methylcatechol in the urine of rats given neomycin sulphate in addition to L-dopa suggests that this metabolite or its precursors arises through the action of intestinal microorganisms. 4-Methylguaiacol is produced *in vivo* from 4-methylcatechol by *O*-methylation in the rat (BAKKE 1970).

Intestinal microorganisms are also involved in the formation of the metabolite with the retention characteristics of *meta*-cresol. However, decarboxylation of phenolic acids with free hydroxyl groups in the *meta* position only has not been shown to be carried out by the intestinal microflora (SCHELINE 1968). The immediate precursor of *meta*-cresol is therefore not known and the possibility that tissue enzymes may also play a part in the pathways leading to this compound from L-dopa remains to be investigated.

Studies on the degradation of L-dopa by mixed cultures of intestinal microorganisms and the possible precursors of the simple phenolic metabolites are in progress in this laboratory.

REFERENCES

Bakke, O. M.: Urinary simple phenols in rats fed purified and nonpurified diets. *J. Nutr.* 1969a, **98**, 209–216.

Bakke, O. M.: Studies on the degradation of tyrosine by rat caecal contents. *Scand. J. Gastroent* 1969b, **4**, 603–608.

Bakke, O. M.: *O*-Methylation of simple phenols in the rat. *Acta pharmacol. et toxicol.* 1970, **28**, 28–38.

O'Gorman, L. P., O. Borud, I. A. Khan & L. R. Gjessing: The metabolism of L-3,4-dihydroxyphenylalanin in man. *Clin. Chim. Acta* 1970, **29**, 111–119.

Sandler, M., F. Karoum, C. R. J. Ruthven & D. B. Calne: *m*-Hydroxyphenylacetic acid formation from L-dopa in man: Suppression by neomycin. *Science N. Y.* 1969, **166**, 1417–1418.

Scheline, R. R.: Drug metabolism by intestinal microorganisms. *J. Pharm.* *
2021–2037.

From the Institute of Pharmacology (Head: Professor Ib Holm-Jensen),
University of Aarhus, Aarhus, Denmark

A Simple Transducer Operating Outflow Recorder

By

F. Nielsen Kudsk

(Received August 5, 1970)

Key-words: Recorder – outflow – method.

The outflow recorder described below was constructed for use in conjunction with the Harvard Apparatus® electronic modular recording system for experiments with perfused, isolated rabbit hearts. Like the well-known recorders described by GADDUM (1929) and ANDREWS (1952) it records the volume of perfusion liquid that flows out in a known interval of time.

The recorder is shown diagrammatically in the figure. The dimensions are not critical and may be estimated from the diagram. The perfusion liquid flows continuously into the recorder through the inlet tube (b). For definite periods of time the outflow from the recorder through the outlet tube (c) is prevented by means of an electro-magnetic valve (Skinner®, type B2 DA9052, not shown in the figure), which may be controlled by an electronic make- and break timing device or a rotary key. The volume of fluid collected while the outflow from the recorder is stopped causes a proportional elevation of a spherical hollow plastic float (e). By means of an attached silver rod (f) the float activates one end of a lever (j) which is fixed to the input shaft of a "Harvard" electronic displacement transducer (i). The DC signal from the transducer is amplified by a "Harvard" electronic recording module, model 350, and the pen response of this module is continuously recorded with regard to time by means of a chart mover. An ordinary potentiometric strip chart recorder can also be used to process the output signal from the transducer. At the end of the fluid collecting period, the electromagnetic valve is opened and the apparatus is emptied to a constant zero level through the outlet tube (c) by means of a water suction pump. The emptying period lasts 1 sec. after which the cycle is repeated.

The apparatus is most easily calibrated by measuring the height of the records at each of a series of different known constant rates of flow. With

the dimensions given in the figure and a liquid collecting period of 10 sec. a linear calibration curve can be obtained within a range of rates of flow from 1 to 55 ml per min. The accuracy within this range is better than ± 0.5 ml/min.

The maximum rate of flow, which can be measured, may be increased by shortening the period of fluid collection. At low rates of flow the sensitivity and accuracy of the apparatus can easily be increased by increasing the electronic amplification of the transducer output voltage. In these cases appropriate calibration curves have to be made.

The apparatus is not sensitive to changes in temperature, fluid viscosity and surface tension and it is not influenced by changes in barometric pressure as opposed to the often used flow meters which depends on a capillary discharge tube and pneumatic coupling to the recording device (STEPHENSON 1948; 1949).

The author is indebted to the chief technician of the institute, Mr. Børge Poulsen, for the practical production of the apparatus.

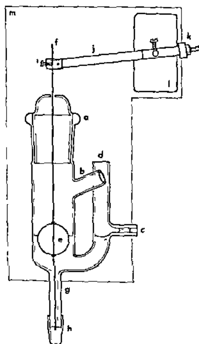


Fig 1. Diagram of the outflow recorder construction: (a) standard borosilicate joint B 24, Quickfit®, (b) inlet tube, (c) outlet tube, (d) air vent, (e) spherical plastic float, 20 mm diameter, (f) silver rod, 1 mm diameter, (g) float centering tube, (h) rubber cap, (i) coupling piece, (j) lever, (k) counterbalance, (l) electronic displacement transducer, Harvard Apparatus®, model 352 and (m) mounting plate.

LETTERS TO THE EDITOR, *Acta pharmacol. et toxicol.* 1971, 29, 110.

REFERENCES

Andrews, W. H. H.: A blood outflow recorder. *J. Physiol.* 1952, **117**, 45–46 P.
Gaddum, J. H.: An outflow recorder. *J. Physiol.* 1929, **67**, Proceedings XVI–XVII.
Stephenson, R. P.: An outflow recorder useful for detecting small amounts of vasopressin. *J. Physiol.* 1948, **107**, 162–164.
Stephenson, R. P.: An apparatus for recording the output and coronary flow in the heart-lung preparation. *J. Physiol.* 1949, **108**, 102–103.

From the Department of Pharmacology and the Section of Toxicology,
University of Copenhagen, Denmark

A Specific Method for the Demonstration of Cannabis Intake by TLC of Urine

By

J. Mørkholdt Andersen, E. Nielsen, J. Schou, A. Steentoft and K. Worm

(Received December 2, 1970)

Key-words: Cannabis – metabolites – urinary excretion.

Our experience with previously published chromatographic methods for the demonstration of cannabis components or metabolites in the urine has shown that false positive reactions occur frequently in control urines.

Hence there is urgent need for developing a specific and dependable method for the qualitative demonstration of cannabis intake by means of urine analysis. As we have recently succeeded in developing such a method we feel it relevant to publish our preliminary findings with regard to its use in forensic problems.

Twenty ml of urine were washed three times with light petroleum (b. p. 40–60°). By this procedure the parent compounds of cannabis (including THC) and unknown compounds are removed from the sample in order to avoid false positive reactions. The main metabolite 7-hydroxy-Δ^1-tetrahydrocannabinol (7-OH-Δ^1-THC) (see NILSSON *et al.* 1970) is retained in the urine, until it is extracted into freshly distilled ether (three extractions) at pH 3.8 (see AGURELL *et al.* 1970). The combined ether extracts are dried with Na_2SO_4, filtered and evaporated to dryness at 37° under nitrogen. To dehydrate the metabolite to cannabinol, 10 mg *p*-toluenesulfonic acid and 50 ml benzene are added and this is followed by boiling for two hours under reflux according to BURSTEIN *et al.* (1970). The benzene solution is then washed with an equal volume of 2 % Na_2CO_3 and dried with anhydrous Na_2SO_4. After adding methanol to 39.5 % (azeotropic mixture) the solution is filtered and evaporated to dryness in a rotating evaporator at 50°.

The residue is applied to a TLC plate and sprayed according to KORTE & SIEPER (1964). A positive reaction is indicated by the occurrence of *two* spots, one with the colour and Rf value like cannabinol, the other like THC (fig. 1). In all the tests the ether extract previous to the addition of *p*-toluenesulfonic acid gave no reaction at spraying. It is therefore concluded that the positive reaction is caused only by the hydration products of the metabolites from the active components.

Fig. 1. Thin-layer chromatogram showing 1) blank control urine, 2) urine showing a positive reaction after cannabis intake and 3) extract of cannabis. The positive reaction is based on the occurrence of *two* spots situated and coloured like cannabinol and THC in the cannabis extract.

Compound	Colour with fast blue salt B	
THC II	brownish violet	
THC I	scarlet	
CBN	violet	
CBD	orange	
	orange	
CBDS	orange	start.
		1. 2. 3.

Symbols:
CBDS = cannabidiol acid
CBD = cannabidiol
CBN = cannabinol
THC = tetrahydrocannabinol

In a blind experiment it was easy to select 7 positive urines from 6 blank control urines (fig. 1). One of the positive urines was a sample from a cannabis smoker, which had been stored at − 20° for more than one year. The other 6 urines were obtained from human volunteers at various times (30 min. to 6 hours) after the oral intake of 750 to 1000 mg of a cannabis resin (approximately 1 % THC).

To get some idea of the time course of the reaction after cannabis intake, 300 mg resin (14 mg THC) was taken orally by a human volunteer. Samples of urine were obtained for 24 hours and fractioned. A significant positive reaction was found during the first 6–7 hours after intake, whilst the test subject reported subjective symptoms for only the first three hours after the cannabis intake.

REFERENCES

Agurell, S., I. M. Nilsson, A. Ohlsson & F. Sandberg: On the metabolism of tritium-labelled Δ1-tetrahydrocannabinol in the rabbit. *Biochem. pharmacol.* 1970, 19, 1333–1339.

Burstein, S. H., F. Menezes, E. Williamson & R. Mechaulam: Metabolism of Δ 1(6)-tetrahydrocannabinol, an active marihuana constituent. *Nature* 1970, 225, 87–88.

Korte, F. & H. Sieper: Zur chemischen Klassifizierung von Pflanzen. XXIV. Untersuchung von Haschisch-Inhaltsstoffen durch Dünnschichtchromatographie. *J. Chromatog.* 1964, 13, 90–98.

Nilsson, I. M., S. Agurell, J. L. G. Nilsson, A. Ohlsson, F. Sandberg & M. Wahlqvist: Δ1-Tetrahydrocannabinol: Structure of a major metabolite. *Science* 1970, 168, 1228–1229.

Acta pharmacol. et toxicol. 1971, 29, 113–126.

From the Department of Pharmacology, Thomas Jefferson University, Philadelphia, Pa. and Office of the Medical Examiner, Department of Public Health, Philadelphia, Pa. U.S.A.

Interfering Peaks in Gas Chromatographic Exclusion Screening of Direct Chloroform Extracts of Blood*

By

S. K. Niyogi and F. Rieders

(Received April 20, 1970)

Abstract: The abilities of 24 endogenous biochemicals to interfere with gas chromatographic exclusion screening analyses for drugs of direct chloroform extracts obtained from forensic blood specimens were studied. The relative retention on OV-17 of the 24 endogenous compounds and the direct extractabilities of 8 of these were compared with those of 30 directly extractable acidic, phenolic, neutral and basic drugs. On direct extracts of blood, gas chromatographic peaks were obtained with phenylethylamine, 5-hydroxymethylfurfural, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, p-hydroxyphenylacetic acid and stearic acid. Palmitic acid and p-hydroxyphenylpropionic acid were not directly extractable, while no gas chromatographic peaks were obtained with three neutral lipids, cholesterol, four cholesterol esters, deoxycholic acid, phosphatidyl-L-serine, heparin and L-glutamine. Tyramine, tryptamine, tristearin and lysolecithin gave gas chromatographic peaks but their direct extractabilities were not determined. Post mortem toxicological cases are described in which apparent barbiturate, tripelennamine, hydroxyglutethimide and desipramine gas chromatographic peaks were probably mimicked by stearic acid, p-hydroxybenzaldehyde and/or tryptamine; differentiations were effected by selective washing of the chloroform extracts with weak and strong bases, acid, ammoniacal $AgNO_3$ and aqueous $NaHSO_3$.

Key-words: Toxicological analysis – ptomaines – gas chromatography – post mortem changes.

In a general search for "poisons" in human tissues and body fluids, endogenous constituents and their breakdown products sometimes either obscure or even mimic significant exogenous agents. Such interferences, with regard to extraction characteristics, alkaloidal color and crystal tests, spectra, solid-liquid chromatography and polarography have been reviewed (TRUHAUT & MOAN 1958; NIYOGI 1970) and are summarized in table 1.

---

* Presented in part at the Twentieth Annual Meeting of the American ... Forensic Sciences, Chicago, Illinois, February 24, 1968.

Table 1.
Biogenic interferants in toxicological analysis.

Interfering compounds	*Interferes with*	Nature of interference	*Reference*	
β-p-hydroxyphenyl-propionic acid	Barbiturate	Color reactions and precipitation test	NICKOLLS	(1951)
β-p-hydroxyphenyl-propionic acid	Non-phenolic substances	Unspecified	LYNCH	(1951)
Natural origin (phenol-like)	Mimics barbiturates	Ultra-violet absorption	CURRY	(1955)
Pseudo-barbiturate	Mimics barbiturates	Ultra-violet absorption	JACKSON & FINKLE	(1963)
Biogenic putrefactive amines	Alkaloids	Color reactions, precipitation tests and paper chromatography	TRUHAUT & MOAN	(1958)
Phenylethylamine	Amphetamine	Ultra-violet absorption and chemical test	GOLDBAUM *et al.*	(1963)
	Atropine, pethidine, dextropropoxyphene, methamphetamine, norephedrine and phenmetrazine	Ultra-violet absorption	GOLDBAUM *et al.*	(1963)
Basic compound (pyridine)	Nicotine or its metabolites	Ultra-violet absorption	GOLDBAUM *et al.*	(1963)
Tyramine	Morphine	Polarography and paper chromatography	KÆMPE	(1964b)
Tryptamine	Commonly occurring alkaloids	Ultra-violet absorption	KÆMPE	(1965b)
Nicotinamide, uracil, thymine	Alkaloids	Ultra-violet absorption	KÆMPE	(1967b)
1-hydroxymethyl-β-carboline	Harman	Ultra-violet-absorption and paper chromatography	KÆMPE	(1967a)
β-phenylethylamine	Amphetamine Amphetamine and pethidine	Color reaction, ultra-violet absorption	KÆMPE	(1965a)
p-hydroxybenzaldehyde	Barbiturate	Ultra-violet absorption	DENTON *et al.*	(1963)

Interfering compounds	Interferes with	Nature of interference	Reference	
Acetaldehyde	Paraldehyde	Unspecified	GOLDBAUM *et al.*	(1963)
Phenol (a preservative in heparin preparations)	Barbiturate	Ultra-violet absorption	GOLDBAUM *et al.*	(1963)
p-hydroxy-phenylethanol	5,5-substituted barbiturates	Ultra-violet absorption	KÆMPE	(1964a)
p-hydroxyphenyl-acetic acid p-hydroxybenzaldehyde p-hydroxybenzoic acid	5,5-substituted barbiturates	Ultra-violet absorption	KÆMPE	(1965a)
5-hydroxymethyl-furfurol	Salicylic acid and barbituric acids	Ultra-violet absorption	KÆMPE	(1965d)

Most of these extractable substances might also be expected to appear in gas-liquid chromatograms obtained with the non-selective columns generally used in forensic toxicology (ANDERS & MANNERING 1967). In this laboratory, exclusion screening gas chromatograms are routinely obtained on direct immiscible, organic solvent-extracts of blood and urine samples in about 2,000 cases involving suspected as well as many presumably natural deaths each year.

By these methods (NIYOGI *et al.* 1965; RIEDERS 1968), using SE-30, QF-1, XE-60, DC-200, OV-101, OV-17 or OV-22, about 10 % of the cases gave "false positive" results. False positives show up as sharp peaks with retention times, some of which correspond to those of foreign compounds. Broad bands are also seen, covering retention intervals in which important drugs are being sought. In these cases, when further analyses show that the apparent or possible foreign compounds are in fact not present, the peaks are most probably due to endogenous biochemicals and/or their breakdown products.

The retention times of these interfering peaks are quite stable, in relation to each other and recur quite consistently. They are prominent in decomposed cases, in about 30 % of crib deaths, in most cases with "milky" serum, and in about 20 % of those traumatic deaths in which there probably was an agonal period of at least several minutes.

The present investigation deals with gas chromatographic characteristics on the moderately polar phenylsilicone OV-17 of 24 common biogenic compounds which appeared to us to be potential interferants in the gas chromatographic exclusion screening procedure briefly described below.

Materials and Methods

Gas chromatographic retentions were determined using the following three per cent solutions of biogenic compounds: Triolein, tripalmitin, trilaurin, tristearin, cholesterol, cholesterol oleate, cholesterol stearate, cholesterol palmitate, cholesterol linoleate, palmitic acid, stearic acid, lysolecithin (all from Sigma Chemical Co., St. Louis, Mo.), 5-hydroxymethylfurfural (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 2-phenylethylamine (Eastman Organic Chemicals, Rochester, N. Y.) were dissolved in chloroform. Deoxycholic acid, tyramine hydrochloride, tryptamine hydrochloride, p-hydroxyphenylacetic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and p-hydroxybenzaldehyde (Eastman Organic Chemicals, Rochester, N. Y.) were dissolved in methanol; p-hydroxyphenylpropionic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and p-hydroxybenzoic acid (Eastman Organic Chemicals, Rochester, N. Y.) were dissolved in acetone. Phosphatidyl-L-serine (Sigma Chemical Co., St. Louis, Mo.) was dissolved in a mixture of methanol and chloroform (1 : 1).

Since heparin (Nutritional Biochemicals Corp., Cleveland, Ohio) and L-glutamine (Sigma Chemical Co., St. Louis, Mo.) are insoluble in these organic solvents, they were injected as solids.

A Research Specialties Co. Model 60-10 and a Model 1600 gas chromatograph, each with flame ionization detector and 5 ft. × ⅛ inch stainless steel columns packed with

Table 2.

Relative retentions of components of standard test mixtures in methanol (see text for gas chromatographic conditions).

Test mixture "A"		Test mixture "B"	
Component	Relative retention	Component	Relative retention
Meprobamate	0.47	Butabarbital (secumalum NFN)	0.91
Amobarbital (pentymalum NFN)	1.00 (= 1.1 minutes)	Pentobarbital (mebumalum NFN)	1.15
Secobarbital (meballymalum NFN)	1.35	Glutethimide	2.18
		Phenobarbital (phenemalum NFN)	3.65

3 % OV-17 on 60/80 mesh Chromosorb G HP (Supelco) were used. Temperatures were adjusted so that relative retentions of the components of two test mixtures (table 2) were similar on both instruments.

The gas chromatographic conditions were as follows:

Sample inlet (injection) temperature:	360°
Column oven temperature reading:	218° – RS Co 60-10
	200° – RS Co 1600
Carrier gas – nitrogen:	60 ml/min, 26 psi inlet pressure

H_2 and air flows were not measured, but were adjusted daily for a maximum response to the test mixture components.

Sample injection: 10 µl Hamilton® liquid syringe and Hamilton® solid injector.

Retentions of the biogenic compounds relative to amobarbital (pentymalum NFN) were determined at an attenuation of 3K with 1 µl liquid samples of the 3 % solutions (i. e. with 30 µg of each agent). In the cases of the insoluble heparin and L-glutamine, as much of the powder as would adhere to the needle tip of the solid injector (approximately 0.5 to 1 mg), which had first been moistened with methanol, was injected. These large sample sizes were used to overcome adsorptive effects and to facilitate operation at an attenuation (3K) which was high enough to give a short and narrow solvent peak so that any short-retention solute peaks would be visible. If no response due to the compound tested was obtained at 3K, the injections were repeated at lower attenuation.

In the present study, extractions from blood were determined as follows: An aliquot of solution containing the compound to be tested was evaporated to dryness in a 4 ml test tube. One ml blank blood and 0.2 ml $CHCl_3$ were added; the tube was inverted 40 times over a period of 1 minute and centrifuged at 3400 rpm for 4 minutes. About 0.1 ml of the chloroform was withdrawn by means of a capillary pipette, transferred to a micro-test tube and 1 µl injected into the gas chromatograph.

The direct extractions by chloroform from blood were not determined for tyramine, tryptamine, tristearin and lysolecithin. These compounds however, did give peaks on direct injection.

The routine procedures for exclusion screening by gas chromatography of direct extracts are as follows:

1. One ml blood, cerebrospinal fluid or homogenates of 1 part tissue and 3 parts water are placed into a 75 × 10 mm test tube, followed by 0.2 ml $CHCl_3$, this was followed by 40 complete inversions in the course of one minute and centrifugation for four minutes at 3400 rpm in a Clay-Adams Sero-Fuge®. As much of the infra-natant chloroform as possible is withdrawn by means of a Pasteur pipette and transferred. Three µl, in one µl increments, are transferred to the tip of a Hamilton® solid injector, each increment being allowed to evaporate before the next increment is added, and the solid residue is then injected into the gas chromatograph. For urine, one ml is acidified with approximately 0.05 ml concentrated HCl and is then treated in the same manner as described for blood. Using columns of 5 ft 3 % DC-200 on Chromosorb W AW/DCMS at 180°, 5 ft 3 % OV-17 on Chromosorb G HP at 200°, or 5 ft 3 % OV-22 on Chromosorb G HP at 230°, and flame ionization detection, approximately 10 ng amounts of barbiturates, dilantin® (phenytoinum NFN), meprobamate and related carbamates, glutethimide, methyprylon, carbromal, ethinamate, propoxyphene, meperidine (pethidinum NFN) and methadone are detectable by this procedure with retention times of less than 10 minutes.
2. Similar extraction on one ml samples are performed after adding approx

0.5 ml concentrated NH_4OH reveals, on the same columns, 10 ng or more of antihistamines, nicotine, methadone, phencyclidine, imipramine, mescaline, as well as the neutrals and bases named above, also with retention times of less than 10 minutes. In addition, on a 5 ft column of OV-101 on Chromosorb G HP at 230°, codeine and phenothiazines in urine are also detectable; their concentrations in and extractions by the present method from other tissues are usually inadequate for detection.

3. For the detection of amphetamine, a one ml sample is mixed as described above with 0.5 ml concentrated NH_4OH, 0.1 ml half saturated aqueous lead acetate and 0.2 ml CS_2. This converts the amphetamine to the isothiocyanate which dissolves in the excess CS_2. Evaporation (at room temperature) of three µl of the CS_2 extract as described above and injection (onto the DC-200, OV-17 or OV-22 columns at the temperatures named) allows the detection of 10 ng amphetamine with a retention time of less than two minutes.

Results

No peaks were obtained from triolein, trilaurin, tripalmitin, cholesterol, cholesterol palmitate, cholesterol oleate, cholesterol stearate, cholesterol linoleate, deoxycholic acid, phosphatidyl-L-serine, heparin and L-glutamine under the conditions described.

Table 3 shows retentions in ascending order, relative to amobarbital (pentymalum NFN), of the remaining biochemicals. These and representative drugs with similar sufficient retentions to be of value, are further subdivided into strong acids, weak acids, neutrals and bases. Brackets around the name of the chemical indicate that the substance showed more than one peak and that the one shown in brackets was of minor importance (usually less than 10 % of the major peak).

Fig. 1 shows the response and separation at an attenuation of X-300 obtained when injecting 1 µl of a mixture in organic solvent containing 13.5 µg each of palmitic acid, stearic acid and 5-hydroxymethylfurfural, 26.1 µg of p-hydroxybenzaldehyde and of amobarbital, as well as 52.2 µg each of 2-phenylethylamine, p-hydroxybenzoic acid, p-hydroxyphenylacetic acid and p-hydroxyphenylpropionic acid. The peak of 5-hydroxymethylfurfural and the major peak of 2-phenyl-ethylamine are within the solvent peak, while the secondary phenylethylamine peak is barely visible as a distortion of the solvent peak's tail. An unidentified peak (9) also appears which may be a Schiff base formed with the acetone or an ester formed with the methanol in the solvent at the high temperature of the injection port.

Fig. 2 shows the result obtained at X-300 when 1 µl of the chloroform extract from the spiked blood sample was injected. The phenylethylamine and 5-hydroxymethylfurfural are again covered by the solvent peak. Palmitic acid and p-hydroxyphenylpropionic acid are not seen; they are evidently insufficiently extracted by the present procedure. The recovery of the other agents was substantial, but by no means complete. The unidentified peak

Table 3.

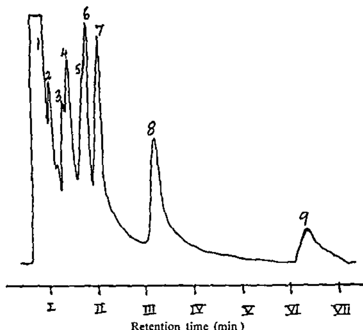
Relative retentions (vs amobarbital = 1.00 [1.1 minute]) of the *endogenous tissue constituents* and of selected drugs with similar relative rententions.

Relative Retention	Strong acids	Weak acids	Neutrals	Bases
0.11	Benzoic acid		Meparfynol® (methylpentynolum NFN)	*(Tryptamine)* (*3) (Hyoscyamine)
0.14		Terpin hydrate	Carbromal	*2-phenethylamine* (*1) *(Tyramine)* (*3) Amphetamine
0.25	Salicylic acid	(Phenylsalicylate)	*5-hydroxymethylfurfural* (*1) (Hydroxyphenamate oxifenamalum NFN)	*(2-phenethylamine)* (Methenamine)
0.39		Metharbital (endiemalum NFN)	*p-hydroxybenzaldehyde* (*1)	Amphetamineisothiocyanate
0.46			Meprobamate	*Tyramine* (*3) Benzocaine (4*)
0 53	*(p-hydroxyphenylacetic acid)*		Carisoprodol (carisoprodatum NFN)	Cocaine
	p-hydroxybenzoic acid (*1)	Nikethamide	Mebutamate	

0.71	*p-hydroxyphenylacetic acid* (*1) (*p-hydroxyphenylpropionic acid*)	Ipral (ethypropumalum NFN)	Methyprylon	Methadone (*4)
0.93	*p-hydroxyphenylpropionic acid* (*2)	Butabarbital	Tybamate	Propoxyphene (*4)
1.29	*Palmitic acid* (*2)	Methohexital (enallynymalum NFN)	Phenacetin	Methamphetamine
1.36		*Lysolecithin* (*3) Secobarbital		*Tyramine* (*3) quinine
1.79				*Tryptamine* (*3) Desipramine
1.86			*Tristearin* (*3) Doxapram	(Imipramine)
2.46	*Stearic acid* (*1)	Mephobarbital (enphenemalum NFN)	Hydroxyglutethimide	Tripelennamine
3.00		Bemegride		*Tryptamine* (*3) Chlorpheniramine

( ) = Minor, secondary (breakdown?) peak.
(*1) = Directly extractable from blood by $CHCl_3$.
(*2) = Not directly extractable.
(*3) = Direct extractability not determined.
(*4) = HCl Salts are soluble in $CHCl_3$ and appear in "Neutral" fraction.

Fig. 1. Gas chromatogram of mixture in mixed solvents.



Injector temperature: 365°
Column oven temperature: 220°
N_2 inlet pressure: 26 psi
Sample: 1 μl, liquid

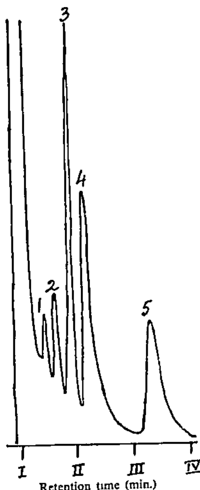
1. Phenylethylamine
2. Para-hydroxybenzaldehyde
3. Para-hydroxybenzoic acid
4. Para-hydroxyphenylacetic acid
5. Para-hydroxyphenylpropionic acid
6. Amobarbital
7. Palmitic acid
8. Stearic acid
9. An unidentified peak

which was seen in fig. 1 (peak No. 9) is not in evidence. This tends to strengthen the methyl ester or acetone-Schiff base formation theory since in the present injection chloroform alone was the solvent.

The following are five illustrative cases to which the exclusion screening procedures were applied.

Case No. 1: A 39-year-old, Caucasian male, with a history of chronic and heavy beer consumption, complained of chest pain while eating and collapsed and died within minutes. Autopsy, performed 18 hours later, with intervening refrigeration of the body at 5°, revealed markedly narrowed coronaries, consistent with coronary insufficiency as the cause of death. A specimen of blood, taken for toxicological exclusion screening, was markedly lipaemic.

Fig. 2. Gas chromatogram of mixture extracted from blood with chloroform.



Injection temperature: 355°
Column oven temperature: 215°
N_2 inlet pressure: 26 psi
Sample: 1 µl, liquid

1. Para-hydroxybenzaldehyde
2. Para-hydroxybenzoic acid
3. Para-hydroxyphenylacetic acid
4. Amobarbital
5. Stearic acid

Case No. 2: An 82-year-old, Caucasian male was found dead at the foot of a flight of cellar stairs in a position and under circumstances indicating that he had tumbled down the steps 4 to 5 hours previously. The cause of death was found to be a broken neck with cord injury including vital reaction. A specimen of aortic blood was taken for toxicological exclusion screening.

Case No. 3: A 70-year-old white male was found dead in bed in a "Skid Row" hotel room. On the basis of age, history, circumstances and results of external examination,

his death was certified as being due to arteriosclerotic cardiovascular disease. Blood was obtained for toxicological exclusion screening by heart puncture through the chest wall.

Case No. 4: A 38-year-old Negro male with a history of alcoholism was found dead in his blood-spattered bathroom. He had last been seen alive four hours previously. An autopsy revealed ruptured oesophageal varices as the cause of death.

Case No. 5: A 60-year-old Caucasian male was found dead in his partly-filled bath tub. He was nude, partly immersed and slightly decomposed. On the basis of circumstances, history and results of an external examination his death was certified as due to natural causes.

In the preceding cases, toxicological exclusion screening consisted of the following examinations of the blood specimens.

1. Micro-diffusion for carbon monoxide, cyanides, fluoride, phenol, phosphorus, sulphide, volatile reductants and volatile oxidants.
2. Head space gas chromatography for volatile organic agents.
3. Ultraviolet spectrometry, thin-layer chromatography, gas chromatography, a fluorescence test for quinine and a microchemical color test for ethchlorvynol and bromides on a direct-chloroform-extract.
4. Trinder's test for salicylates.
5. A Reinsch test for heavy metals.

The results of the gas chromatographic analyses performed on the direct chloroform extracts and the conclusions drawn are summarized in table 4.

Discussion

The endogenous compounds which were extractable and showed up on the gas chromatograms gave peaks sufficiently large and sharp to be mistaken for significant concentrations of the drugs with similar retentions. Differentiations between the biochemicals and their gas chromatographic "drugmates" can often be effected by selectively washing out from the chloroform one or the other. By washing the chloroform extract briefly with an equal volume of 2 % fresh $NaHCO_3$ and then injecting a fresh aliquot of this washed chloroform, the peak will have disappeared or at least very markedly diminished, providing it was due to one of the strong acids, but will appear essentially unaltered if it was due to one of the other groups of compounds. Thus salicylic acid can be differentiated from 5-hydroxymethylfurfural; p-hydroxybenzoic acid from nikethamide and mebutamate. Similarly, brief washing with 0.3 N-NH_4OH removes both weak and strong acids, while more prolonged shaking with 1 N-NaOH also accomplishes this and in addition, hydrolyzes or degrades esters as well as some other drugs such as amphetamine, benzocaine, cocaine, bemegride and glutethimide; thus either altering their retention or removing them. A wash with 0.1 N-H_2SO_4 removes most of the bases, while a wash with dilute, nearly neutral ammoniacal silver nitrate

Table 4.

Relative retentions in gas chromat[illegible]ams of direct $CHCl_3$ blood extracts in five illustrative cases (see text for histories)

Case No.	Relative retentions	Possible compounds and concentrations (mg/100 ml)		Effects of washings on peak height						Most probable identity of compounds
				$NaHCO_3$	NH_4OH	NaOH	H_2SO_4	$AgNO_3$ NH_4OH	$NaHSO_3$	
1	2.5	Mephobarbital	0.5	80 % W	W	W	O	O	O	Stearic Acid
		Hydroxyglutethimide	0.2							
		Tripelennamine	0.9							
		Stearic acid								
2	0.4	Metharbital	0.05	O	O	O	O	O	W	p-Hydroxybenzaldehyde
		p-hydroxybenzaldehyde								
	2.4	Mephobarbital etc. (See Case 1)	0.22	W	W	W	O	O	O	Stearic Acid
3	0.4	Metharbital	0.06	O	O	O	O	O	W	p-Hydroxybenzaldehyde
		p-hydroxybenzaldehyde								
	1.4	Secobarbital	0.04	slight W	most W	W	slight W	Not done	slight W	Not secobarbital (on basis of UV & TLC)
		Lysolecithin								
	2.4	Mephobarbital etc. (See Case 1)	0.07	W	W	W	O	O	O	Stearic Acid
4	0 4	Metharbital	0.17	O	O	O	O	O	W	p-Hydroxybenzaldehyde
5	0.4	Metharbital	0.50	O	O	O	O	O	W	p-Hydroxybenzaldehyde
		p-hydroxybenzaldehyde								
	0 9	Butabarbital	0 07	slight W	most W	most W	O	O	O	Not a barbiturate (on basis of UV & TLC)
	1.8	Desipramine	0.2	O	O	O	W	70 % W	O	Tryptamine
	2.4	*Mephobarbital* etc. (See Case 1)	0.3	W	W	W	O	O	O	Stearic Acid

O = Did not wash out.
W = Washed out.

removes some of the biogenic amines but not most other bases. Dilute (1 to 3 %) $NaHSO_3$ washes out 5-hydroxymethylfurfural.

These techniques are analytically valid for differentiation by chemical exclusion and characterization, using gas chromatography as an efficient separation procedure and as an exquisitely sensitive detection method. The illustrative cases present emphasize again that the test gas chromatographic retention values per se, although constituting one characteristic of a compound, do not of course, constitute identification in most instances. At the same time, they show the value of simple chemical manipulation of extracts to make gas chromatographic results more meaningful.

Acknowledgement

The National Institute of Law Enforcement and Criminal Justice provided financial support for manuscript preparation. The views reported in the manuscript do not necessarily indicate the concurrence of the Institute in the statements or conclusions contained therein.

REFERENCES

Anders, W. M. & G. J. Mannering: *Progress in chemical toxicology.* Vol. 3. Ed.: Stolman, A. Academic Press Inc., New York, 1967, p. 121–218.

Curry, A. S.: Interference to the ultra-violet spectrophotometry of barbiturates. *Nature* 1955, **176**, 877–878.

Denton, S, N. Dunnett & H. J. Walls: Para-hydroxybenzaldehyde in human viscera. *Nature* 1963, **198**, 793–794.

Goldbaum, L. R., E. H Johnston & J. M. Blumberg: The practice of identification in analytical toxicology. *J. Forensic Sci.* 1963, **8**, 286–294.

Jackson, J. V. & B. S. Finkle: Occurrence of pseudo-barbiturates in postmortem material. *Nature* 1963, **199**, 1061–1063.

Kæmpe, B.: Interfering substances by determination of poisons in autopsy material. I. p-hydroxyphenylethanol *Acta pharmacol. et toxicol.* 1964a, **21**, 326–332.

Kæmpe, B.: Interfering substances by determination of poisons in autopsy material. II. Tyramine. *Acta pharmacol. et toxicol.* 1964b, **21**, 333–338.

Kæmpe, B.: Interfering substances by determination of poisons in autopsy material. III *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde. *Acta pharmacol. et toxicol.* 1965a, **22**, 83–92.

Kæmpe, B.: Interfering substances by determination of poisons in autopsy material. IV. Tryptamine. *Acta pharmacol. et toxicol* 1965b, **22**, 126–130.

Kæmpe, B.: p-hydroxyphenylacetic acid, β-phenylethylamine and tyramine in postmortem livers stored under different conditions. *Acta pharmacol. et toxicol.* 1965c, **23**, 15–26.

Kæmpe, B.: Interfering substances by determination of poisons in autopsy material. V. 5-hydroxymethylfurfurol. *Acta pharmacol. et toxicol.* 1965d, **23**, 360–364.

Kæmpe, B.: Interfering substances in the determination of poisons in autopsy material: 1-hydroxymethyl-β-carboline. *Acta pharmacol. et toxicol.* 1967a, 25, 155–161.
Kæmpe, B.: Interfering substances in the determination of poisons in autopsy material: Nicotinamide, uracil and thymine. *Acta pharmacol. et toxicol.* 1967b, 25, 249–257.
Lynch, G. R.: Identification of β-p-hydroxyphenylpropionic acid in visceral extracts. *Analyst* 1951, 76, 610–611.
Nickolls, L. C.: Occurrence of β-p-hydroxyphenylpropionic acid in viscera. *Analyst* 1951, 76, 609–610.
Niyogi, S. K.: Methods of separation of drugs from biological materials: A quantitative evaluation. *J. Forensic Med.* 1970, 17, 20–41 & 1970, 17, 72–95.
Niyogi, S. K., V. F. Cordova & F. Rieders: Detection of a secobarbital metabolite in human liver by gas chromatography. *Nature* 1965, 206, 716–717.
Rieders, F.: Gas chromatographic analysis in toxicology – Application to bioanalytical exclusion screening. In: *Theory and application of gas chromatography in industry and medicine.* Ed.: H. S. Kroman and S. R. Bender. Grunn and Stratton, New York and London, 1968, 266–273.
Truhaut, R. & G. le Moan: Sur l'interference des ptomaines dans l'identification des alcaloides en toxicologie medicolegale. *Ann. méd. lég.* 1958, 38, 394–420.

Acta pharmacol. et toxicol. 1971, 29, 127–134.

From the Institute of Physiology, University of Odense,
Odense, Denmark

Acute *in Vitro* Effect of Progesterone Dissolved in Dimethyl Sulphoxide on Isotonic Contractions and Electrical Potentials of Isolated Uteri from Early Pregnant or Hormone Pre-Treated Rats

By

N. Elner-Jensen

(Received June 9, 1970)

Abstract: Spontaneous isotonic contractions and electrical activity were obtained from 3 cm sections of rat uteri placed in a 6 ml organ bath. The electrical activity was recorded by means of 2 pairs of platinum wire electrodes, each pair measuring the activity over a 2 to 4 mm uterus section. Uteri were used from 21 pregnant rats (length of pregnancy was 1 to 8 days) and from 4 non-pregnant rats pre-treated for 6 or 8 days with 10 mg/kg progesterone subcutaneously. When 0.17 vol % dimethyl sulphoxide (DMSO) was added to the organ bath no or only small changes in the electrical activity and the spontaneous contractions of the myometrium were observed. Administration of progesterone (83 µg per ml organ bath) dissolved in DMSO (50 mg/ml) inhibited the contractions after 10 to 60 seconds, but no or only minor changes were registered in the electrical activity. After DMSO-progesterone solution had been washed out, the contractions reappeared after 7 to 20 minutes.

Key-words: Progesterone – DMSO solvent – uterus contractions – *in vitro* – rats.

Dimethyl sulphoxide (DMSO) is a colourless, nearly odourless fluid miscible with water. Owing to its ability to penetrate into tissues, to transport steroids through the skin and because of its postulated anti-inflammatory effect, DMSO was extensively investigated and used in the years 1965 to 1967 (Weyer 1967). Because of its low toxicity and capacity to dissolve steroids, it was used in the *in vivo* screening of steroids and non-steroidal substances for possible anti-cancer effects (Rosenkrantz *et al.* 1963).

The aim of the present investigation was to evaluate the possibility of using DMSO in experiments on the isolated uterus *in vitro* under conditions when DMSO produced no effect on contractions and electrical potentials; the possibility of inducing an acute effect of progesterone on the uteri when dissolved in DMSO was also investigated.

Method

Three to six month old Wistar female rats were used for the experiment. Before the experiment the animals had free access to a commercial rat pellet diet and tap water in an animal house at a temperature of 23° and a relative humidity of 50 to 70 %. In order to get pregnant animals, 5 females were caged together with one male and vaginal smears were taken every morning. The day on which sperms were observed in the vagina was termed day 0, and at this stage the animals were considered to be pregnant and the length of the pregnancy was calculated from this day. 21 animals with durations of pregnancies of one to eight days were used for one part of the investigation. Another four intact non-pregnant rats were injected subcutaneously daily with 10 mg/kg progesterone dissolved in olive oil for 6 or 8 days before sacrifice (table 1).

After decapitation of the rat a 3 cm section of one of the uterine horns was placed in a dish with Ringer solution at 37° (THORP 1962). 2 pairs of platinum wire electrodes were drawn through the myometrium at a distance of 2 to 4 mm between them. The organ was then placed in a 6 ml organ bath, one end being connected with a transducer for isotonic registration (fig. 1). The uterus was loaded with 2.0 to 2.5 g, and the electric potentials were registered by means of a DISA differential amplifier to an oscilloscope and a Mingograph 34, where the contractions were also registered. The organ bath was kept at 37° by means of a thermostat and a gas mixture (95 % O_2, 5 % CO_2) was bubled through the Ringer solution.

The uterus was allowed to recover for 20 min. before the start of the registration. First a control period of 5 to 10 min. was registered before 0.01 ml DMSO was added (~ 0.17 vol % in the bath). After 2 minutes the mixture was changed to pure Ringer solution. Two minutes later a new change to pure Ringer solution was made. Then 0.01 ml of a solution of 50 mg progesterone per 1 ml DMSO (~ 83 μg/ml bath) was added. After 2 minutes the solution was again changed twice after which the experiment was terminated. Six of the experiments were continued with registration of short periods during the next half hour.

In 5 cases 0.01 ml pure DMSO was added and separated 4 times by double washings to control the effect of repeated DMSO treatments

No quantitative analysis of electric frequency was used to assess the potentials. Hamilton syringes with 100 μl volume were used for the administration of DMSO and DMSO-progesterone solutions.

Results

The registered electric activity consisted of single spikes or trains of up to 10 sec. duration (fig. 2). Between these vigorous discharges a background activity or electrical silence was observed. In each experiment the contractions of the uteri were observed at intervals of 20 to 70 sec., the intervals being almost constant.

In some of the experiments the electrical impulses were synchronous on both channels, in others asynchronous but a mixture of the 2 patterns was also registered. The synchronous and asynchronous registrations, rarely changed during an experiment. The potentials and the contractions were not,

Table 1.

The effect of DMSO and DMSO-progesterone on the isolated uterus. In the first part of the table DMSO treatment was repeated four times.

Pretreatment or day of pregnancy	Control period		DMSO		DMSO-progesterone	
	Contractions	Spikes	Contractions	Spikes	Contractions	Spikes
			(Four periods)			
–	++	++	++	{++ / +		
–	+	++	+	+		
–	++	++	{++ / +	++		
–	++	++	++	++		
–	++	++	++	++		
10 mg/kg progesterone per day						
×6	++	++	++	++	[illegible]	[illegible]
×6	++	++	++	++	[illegible]	[illegible]
×8	++	++	++	++	[illegible]	[illegible]
×8	++	++	++	++	[illegible]	[illegible]
Day of pregnancy						
1	+	++	+	++	[illegible]	[illegible]
1	+	++	+	++	[illegible]	[illegible]
1	++	+	0	+	[illegible]	[illegible]
2	++	++	++	++	[illegible]	[illegible]
2	++	++	++	++	[illegible]	[illegible]
2	++	+	++	+	[illegible]	[illegible]
2	+	++	+	++	[illegible]	[illegible]
3	++	++	++	++	[illegible]	[illegible]
3	++	++	++	++	[illegible]	[illegible]
3	+	++	+	++	[illegible]	[illegible]
4	++	++	++	++	[illegible]	[illegible]
4	+	++	+	++	[illegible]	[illegible]
4	++	++	++	++	[illegible]	[illegible]
5	++	++	++	++	[illegible]	[illegible]
5	++	++	++	++	[illegible]	[illegible]
6	+	+	+	+	[illegible]	[illegible]
6	++	+	++	+	[illegible]	[illegible]
6	+	++	++	+	[illegible]	[illegible]
8	++	+	++	+	[illegible]	
8	++	+	++	+	[illegible]	
8	++	++	++	[illegible]	[illegible]	

The effects are graduated from 0 (no electrical acti[illegible] represents an intermediate stage. The electrical activit[illegible] 3 cases, the contractions ++ except in the DMSO[illegible] contractions appeared.

as a rule, synchronous, but a train of potentials was often seen at the beginning of or during the contraction phase. When this occurred it remained constant during the whole experiment.

The repeated administration of DMSO 4 times did not change the contractions of the uterus or the electrical potentials except in 2 cases where a decrease in the numbers of potentials and the degree of contractions, respectively, was observed during 25 changes in the 5 control uteri (table 1).

Four uteri from intact rats injected with progesterone showed, after the recovery period, vigorous contractions and electrical activity. When DMSO-progesterone was added, the potentials were unchanged, while the contractions ceased.

Out of 21 rats considered to be pregnant day 1, 2, 3, 4, 5, 6 or 8, strong contractions were registered from 14, while 7 (equally distributed in the material) showed weaker contractions. In 15 rats the electrical activity was comparable to that of the non-pregnant animals, and in 6 animals of various lengths of pregnancy the frequency of impulses was somewhat diminished. In 18 cases the contractions of the uteri disappeared when DMSO-progesterone was added (fig. 2). The electrical activity was unchanged in 19 cases after the

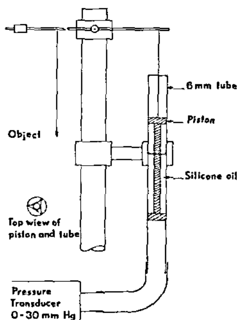


Fig. 1. Isotonic transducer. During contraction of the uterus the piston moves upwards and the oil surface is lowered in the tube. The hydraulic pressure difference is transduced by the pressure transducer, the output of which is fed to a recorder (constructed by chief engineer Ole Johansen, Institute of Physiology, Odense).

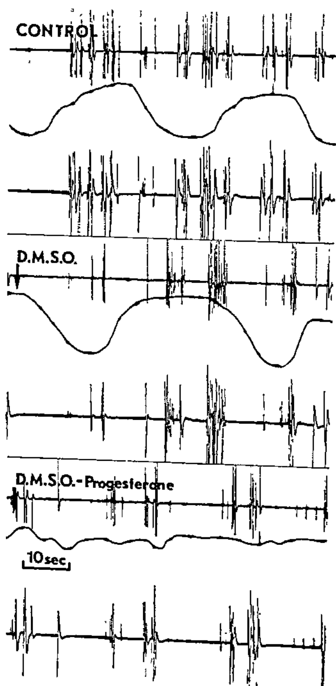


Fig. 2. The *in vitro* effect of DMSO and DMSO-progesterone on the isolated uterus of a four day pregnant rat. The upper and lower traces (a, c) are from the electrodes, the middle trace (b) from the isotonic force transducer.

addition of DMSO-progesterone, while it increased in two, diminished in one, and disappeared in one case.

When DMSO-progesterone was added, no or only one contraction was observed, a full effect being obtained after 10 to 60 sec. (fig. 2).

In 6 cases we investigated whether the effect of DMSO-progesterone was reversible. In 5 of these cases the contractions of the uterus started 7 to 20 min. after the second washing.

Discussion

The experiments indicated that DMSO concentrations of 0.17 vol % are tolerated by uteri in this type of *in vitro* experiment as indicated by FARRANT (1965), even when the treatment is repeated. They also showed an almost instantaneous (less than one min.) and reversible effect of progesterone.

CSAPO (1954) showed that the contractions in an isolated electrically stimulated rabbit uterus diminished if progesterone was added to the organ bath. KNIFTON (1966) showed that the tension in an isolated pig uterus diminished both during spontaneous contractions and during electrically induced contractions, if progesterone was added to the organ bath. He used a "watersoluble" progesterone (Intravenous Primulut). BYGDEMAN & ELIASSON (1964) showed that progesterone in an aqueous vehicle containing propylene glycol and urethane inhibited spontaneous contractions in the human myometrium. In a bath containing 20 µg progesterone/ml the contractions were diminished to about half and in some of the strips up to 100 %. In a bath containing 60 µg progesterone per ml the spontaneous contractions were inhibited by 100%. PHARRISS & RUSSELL (1968) showed that 20 µg progesterone per ml dissolved in 3 % ethanol or 0.06 % albumin carrier could inhibit spontaneous contractions in an organ bath. PINTO *et al.* (1967) showed that 500 to 2500 µg progesterone per ml in an organ bath blocked the action of oxytocin on the internal and medial layer of the myometrium, while in the external layer it reduced the intensity but increased the frequency of the contractions. In contrast to our results they stated that the progesterone block was not reversible.

Our dose (83 µg progesterone per ml organ bath) for the inhibition of contractions is in good agreement with those of KNIFTON (1966) and PHARRISS & RUSSELL (1968). The doses of BYGDEMAN & ELIASSON (1964) cannot be compared with our doses, since continuous infusion with progesterone was used. With the technique used by BYGDEMAN & ELIASSON (1964) it was not possible to estimate how rapidly the effect occurred and KNIFTON (1966) does not mention how quickly he could register the effect. PINTO *et al.* (1967) mentioned that the spontaneous contraction disappeared "very quickly" but

did not state the time in minutes. Our experiment therefore seems to be the first which describes an effect of progesterone on an isolated uterus within less than one minute. KURIYAMA(1961) observed a termination of contractions in post partum mouse uteri 8 min. after treatment with 20 μg/ml progesterone. The rapid effect in our experiment compared to these, may be attributed to the DMSO used as solvent.

CSAPO *et al.* (1963) have shown synchronization between potentials registered from 3 different uterine segments and pressures in the uterine lumen in a rabbit in labour stimulated by the administration of 100 μ units of oxytocin. A similar synchronisation between contractions and electrical activity was seldom seen in our experiments with isolated uteri from non-pregnant progesterone-treated or early pregnant animals. Because of differences in the duration of pregnancy, in the intrauterine pressure, in different species and *in vivo* as compared to *in vitro* experiments, it is not yet possible to explain the difference.

The electrical activity of the uterus was only estimated by means of a Mingograph registration. The potentials were recorded by platinum wires as the potential difference over a 2 to 4 mm uterus segment. Here the total electrical activity was almost unchanged after progesterone treatment although the contractions stopped. This might indicate that there is a failure of co-ordination between neurones and that the single neurone is responsible for its own rhythm. The solution of the problem, however, needs further investigation.

REFERENCES

Bygdeman, M. & R. Eliasson. Effect of progesterone and oestrone on the motility and reactivity of the pregnant human myometrium *in vitro*. *J. Reprod. Fertil.* 1964, **7**, 47–52.

Csapo, A.: Dependence of isometric tension and isotonic shortening of uterine muscle on temperature and on strength of stimulation. *Am. J. Physiol.* 1954, **177**, 348–354.

Csapo, A., H. Takeda & C Wood. Volume and activity of the parturient rabbit uterus. *Am. J. Obst. & Gynec.* 1963, **85**, 813–818.

Farrant, J.. Mechanism of cell damage during freezing and thawing and its prevention. *Nature* 1965, **205**, 1284–1287.

Kuriyama, H.: The effect of progesterone and oxytocin on the mouse myometrium. *J Physiol.* 1961, **159**, 26–39.

Knifton, A.: The influence of the female sex hormones on the response of the pig uterus to electrical stimulation. *J. Pharm. Pharmac.* 1966, **18**, Suppl., 151S–159S.

Pharriss, B. B. & R. L. Russell: A comparison of progesterone and epinephrine inhibition on the myometrium of the rat. *Biochem. Pharmacol.* 1968, **17**, 355–362.

Pinto, R. M., U. Lerner & H. Pontelli: The effect of progesterone on oxytocin induced concentration of the three separate layers of human gestational myometrium in the uterine body and lower segment. *Am. J. Obst. & Gynec.* 1967, **98**, 547–554.

Rosenkrantz, H., Z. Hadidian, H. Seay & M. M. Mason: Dimethylsulfoxide: its steroid solubility and endocrinologic and pharmacologic-toxicologic characteristics. *Cancer Chemotherapy Reports* 1963, **31**, 7–24.

Thorp, R. H.: Posterior Pituitary Hormones. In: *Methods in hormone research.* Ed. R. I. Dorfman. Academic Press, London. 1962, 500.

Weyer, E.: Biological actions of dimethylsulfoxide. *Ann. N. Y. Acad. Sci.* 1967, **141**, 1–671.

Acta pharmacol. et toxicol. 1971, 29, 135–144.

From the Department of Pharmacology, University of Göteborg, Göteborg, Sweden

The Action of Dopamine on Arterial Blood Pressure in the Rat

By

A. Rubenson

(Received October 16, 1970)

Abstract: The mean arterial blood pressure in conscious normotensive Sprague-Dawley rats was recorded by means of in-dwelling arterial catheters. Dopamine hydrochloride (DA) was infused intravenously from 0.04 µg/min. up to 0.22 µg/min. The infusions always resulted in a hypertensive reaction. Intravenous injections of DA (1–50 µg/kg) or noradrenaline bitartrate monohydrate (NA) (0.02–0.5 µg/kg) were given before and after phenoxybenzamine (PBZ) (5 mg/kg); protriptyline (PTP) (10 mg/kg) and nialamide (100 mg/kg). The DA injections alone resulted in a pressor action. PBZ blocked or considerably diminished the pressor action of DA. PTP did not result in a clear-cut augmentation of the blood pressure response to DA like that seen after the various equipotent NA doses tested. Furthermore, there was no prolongation of the duration of the pressor action of DA after PTP, though this was found after NA. Nialamide did not alter the magnitude of the pressor action of DA and NA. However, the hypertensive response of L-DOPA (25 mg/kg) was markedly augmented by pretreatment with nialamide (10 mg/kg).

Key-words: Blood pressure – dopamine.

In a previous publication it was shown that the injection of L-DOPA into the rat produced a hypertensive response, which could be blocked by pretreatment with a peripheral DOPA-decarboxylase inhibitor (Henning & Rubenson 1970a & b). This finding strongly indicated that the hypertensive reaction was of peripheral origin and mediated via the DOPA metabolites dopamine (DA) or noradrenaline (NA) or both. Large amounts of DA were found in the peripheral tissues after the injection of L-DOPA; this accumulation was prevented by the decarboxylase inhibitor (Henning & Rubenson 1970a).

The action of DA on the blood pressure could occur e.g. through direct stimulation of adrenergic receptors or indirectly via the formation of NA or by displacement of endogenous NA from the sympathetic nerve endings.

There is evidence from previous studies for both direct and indirect effects of DA on various organs (FLECKENSTEIN & BURN 1953; TSAI *et al.* 1967; SPIERS & CALNE 1969). Furthermore, the influence of DA on the blood pressure is complicated and dependent on the species. Both pressor and depressor actions have been reported (HOLTZ & CREDNER 1942; HORNYKIEWICZ 1958; HORNYKIEWICZ & OBENAUS 1958; GOLDBERG & SJOERDSMA 1959; MAXWELL *et al.* 1960).

In an attempt to elucidate the mechanism of the blood pressure response to L-DOPA in rats we have now investigated the blood pressure response to DA in this species. Experiments were set up in which small doses of DA were infused intravenously under continuous blood pressure recording. Furthermore the influence of intravenous injections of DA on the blood pressure was studied in some basic experiments and under three different conditions: (1) blockade of adrenergic α-receptors; (2) inhibition of the active amine concentrating mechanism in the sympathetic nerves; (3) inhibition of monoamine oxidase.

Methods

Male Sprague Dawley rats weighing 250–300 g were used. Under pentobarbitone sodium (mebumalum NFN) anaesthesia (30–40 mg/kg intraperitoneally) polyethylene catheters were inserted into the left carotid artery (HENNING 1969), and into the right external jugular vein. The free end of the venous catheter was passed subcutaneously between the eye and the ear on the right side of the neck and exteriorized together with the arterial catheter. The mean arterial blood pressure was recorded in conscious unrestrained rats placed in separate cages. Statham P23 Dc pressure transducers writing on a Grass Model 7 Polygraph were used. As a rule, recordings were not performed until 2 days after the operation.

All drugs were given via the venous catheter. In some of the experiments the catheters were connected to motor-driven syringes delivering dopamine hydrochloride dissolved in isotonic saline at constant rates for ten-minute periods separated by ten-minute intervals (for details see Results). During the intervals, the blood pressure of the animals always returned to their basal level. The blood pressure response after the various DA doses infused was measured as the difference between the maximal peak-change during the ten-minute period of intravenous infusion and the average of the mean arterial blood pressure 2–3 minute before the beginning of the infusion. A dose-response curve for the increase in blood pressure was obtained. In other experiments the arterial pressure was recorded continuously and increasing doses of DA or l-noradrenaline bitartrate monohydrate were given by intravenous injection into the same rat. Each dose was given twice to eliminate the dead space of the venous catheter, and the second response was used for calculation. All the doses of dopamine and noradrenaline were given in a volume of 1 ml/kg. This volume of saline alone had no effect on the blood pressure. The dose-response curves were obtained before and after pretreatment with phenoxybenzamine (bensylytum NFN) (PBZ) (5 mg/kg), protriptyline hydrochloride (PTP) (10 mg/kg) and nialamide (100 mg/kg). The various doses tested were given at least five minutes apart and never before the animals had retained a

steady mean arterial blood pressure. The blood pressure responses were measured as the difference between the maximal peak change and the averages of the recordings for the one minute period immediately before the administration of the drug. The basal blood pressure prior to testing of DA and NA was calculated as the average of 10 minute-periods immediately before the injection of the first dose of DA in the different experimental series.

The duration of the mean arterial blood pressure response after the various dopamine and noradrenaline doses was measured as the time interval between the beginning of the alteration and the time of regression of the blood pressure to a basal steady level.

In one group of experiments the mean arterial blood pressure was recorded after intraperitoneal injection of L-DOPA and nialamide (for details see Results). The alterations in blood pressure in these experiments were measured as the difference between the averages of recordings for five-minute-periods immediately before the administration of the drugs and at various subsequent time intervals (for details see Results).

The doses of all the compounds used in the experiments were based on the weight of the salt. Tests of significance were carried out by Student's t-test and analysis of variance with two independent criteria of classification. P values less than 0.05 were regarded as significant.

Results

Dopamine infusion.

Four animals were given increasing doses of dopamine by intravenous infusion as described in Methods at a constant rate: 0.04 μg/min. (0.02 ml/min.), 0.22 μg/min. (0.11 ml/min.), 1.32 μg/min. (0.062 ml/min.), 4 μg/min. (0.20 ml/min.), 7 μg/min. (0.11 ml/min.), 12.4 μg/min. (0.02 ml/min.), 22μg/min. (0.11 ml/min.). The average changes in mean arterial blood pressure are shown in fig. 1 and as can be seen, all the doses tested resulted in a hypertensive response. Infusion of isotonic saline in the same volumes and at the same rates was given to two animals and no changes in blood pressure were observed.

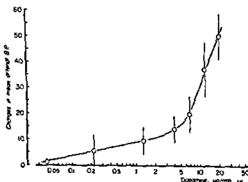


Fig. 1. Average changes in mean arterial blood pressure seen after intravenous infusion of increasing doses of dopamine hydrochloride (n = 4). The symbols are means with S. E. M.

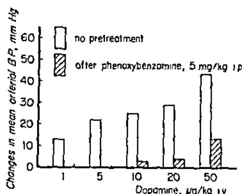


Fig. 2. Average changes in mean arterial blood pressure after intravenously injected dopamine hydrochloride before (open symbols, n = 3) and 1 hour after administration of phenoxybenzamine (PBZ) (5 mg/kg) (shaded symbols, n = 3). The values represent the means. PBZ pretreatment abolished or significantly ($P < 0.001$) prevented the pressor action of DA. P values were obtained by analysis of variance. Error variance = 17.8.

Phenoxybenzamine (PBZ) series.

The PBZ administration (5 mg/kg intraperitoneally) did not influence the mean arterial blood pressure (basal BP 107 mm Hg, S. E. M. 3.8, BP 1 hr after PBZ 104 mm Hg, S. E. M. 2.33).

Three animals were injected intravenously with DA 1, 5, 10, 20, 50 µg/kg before and after PBZ. This pretreatment abolished the hypertensive response to 1 and 5 µg/kg and significantly reduced the magnitude of the pressor response to DA 10, 20, 50 µg/kg ($P < 0.001$) (see fig. 2).

Protriptyline (PTP) series.

The PTP administration (10 mg/kg intraperitoneally) did not influence the mean arterial blood pressure. (First series: Basal BP 133 mm Hg, S. E. M. 3.2; BP 1 hr after PTP 136 mm Hg, S. E. M. 6.0; Second series: Basal BP 136 mm Hg, S. E. M. 4.2; 2 hr after PTP 134 mm Hg, S. E. M. 8.1).

Dopamine: Five animals were given intravenous injections of DA in increasing doses 1, 5, 10, 20, 50 µg/kg. The changes in blood pressure and the duration for the doses 20 and 50 µg/kg are shown in fig. 3 and table 1, respectively.

Pretreatment with PTP 10 mg/kg had no significant effect on the magnitude or the duration of the blood pressure response to DA, except after 10 µg and 50 µg/kg. These doses of DA produced a significantly larger increase in blood pressure ($P < 0.025$; $P < 0.05$ respectively) (see fig. 3).

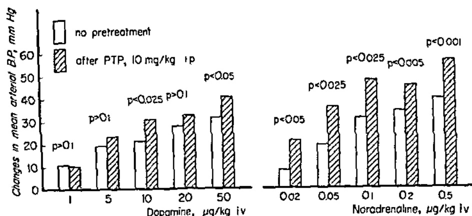


Fig. 3. Average changes in mean arterial blood pressure after the intravenous injection of various doses of dopamine hydrochloride and l-noradrenaline bitartrate monohydrate before (open symbols) and 1–2 hours after administration of protriptyline 10 mg/kg (shaded symbols). The values given are the means. P values were obtained by analysis of variance. First series of experiment: DA 1, 5, 10, 20 and 50 μg/kg followed by NA 0.2 and 0.5 μg/kg (n = 5). Error variances: DA = 36.5; NA = 26 0. Second series of experiment: NA 0.02, 0.05 and 0.1 μg/kg (n = 3). Error variance: 45 6.

Noradrenaline: Five animals were given intravenous injections of NA 0.2 μg and 0.5 μg/kg before and after PTP 10 mg/kg intraperitoneally (see fig. 3). The PTP pretreatment caused a significant augmentation of the hypertensive response as compared to that after NA alone both with respect to magnitude and duration (amplitude: $P < 0.01$; $P < 0.001$, respectively; duration: $P < 0.001$).

Table 1.

	DA 20	DA 50	NA 0.2	NA 0.5
Controls	46A	77C	62E	86G
1–2 hrs after PTP 10 mg/kg	70B	120D	173F	252H

A–B: $P > 0.1$
C–D: $P > 0.1$
E–F: $P < 0.001$
G–H: $P < 0.001$

Average durations of the hypertensive response to various doses of dopamine hydrochloride and l-noradrenaline bitartrate monohydrate before and 1–2 hours after administrations of protriptyline 10 mg/kg (PTP). The values are means given in seconds. P values were obtained by analysis of variance. Error variance: in the dopamine series 144 8 and in the noradrenaline series 365.8.

Since the effects of lower doses of DA were not significantly augmented by the PTP pretreatment it became of interest to test doses of NA, which produced hypertensive responses equivalent to DA 1 and 5 μg/kg. Therefore NA 0.02, 0.05 and 0.1 μg/kg was tested in 3 animals before and after PTP 10 mg/kg. The pressor response seen after these doses was significantly augmented by the PTP pretreatment ($P < 0.05$, $P < 0.025$, $P < 0.025$).

Nialamide series.

The nialamide administration alone did not influence the mean arterial blood pressure (Basal BP 113 mm Hg, S. E. M. 7.9; BP 1 hr after nialamide 101 mm Hg, S. E. M. 11.0).

Dopamine.

Three animals were injected intravenously with DA in increasing doses 1, 5, 10, 20 and 50 μg/kg before and after nialamide 100 mg/kg. All dopamine doses given produced a hypertensive response (see fig. 4). The nialamide pretreatment did not significantly influence the blood pressure response to the different doses of DA tested.

Noradrenaline.

Three animals were given a single dose of NA 0.2 μg/kg before and after nialamide 100 mg/kg. This pretreatment did not significantly influence the hypertensive reaction (see fig. 4).

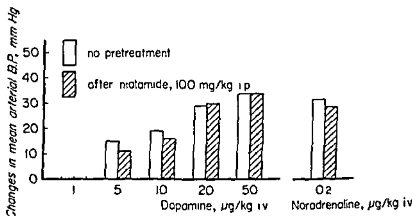


Fig. 4. Average changes in mean arterial blood pressure after the intravenous injection of various doses dopamine hydrochloride and l-noradrenaline bitartrate monohydrate before (open symbols, $n = 3$) and 30 minutes after administration of nialamide 100 mg/kg (shaded symbols, $n = 3$). The values given are the means. The nialamide pretreatment did not significantly change the magnitude of the blood pressor response to dopamine ($P > 0.1$; analysis of variance) or noradrenaline ($P > 0.1$; t-test). Error variance in the dopamine series 3.6.

DOPA.

Intraperitoneal injection of L-DOPA, 25 mg/kg, alone had no effect on the blood pressure. The mean values recorded before and 20 minutes after administration were 106 mm Hg (S. E. M. 4.3, n = 4) and 110 mm Hg, respectively (S. E. M. 4.3, n = 4), P > 0.1.

Two hours later the same animals were given an intraperitoneal injection of nialamide (10 mg/kg). Nialamide only did not seem to influence the blood pressure. The mean blood pressure values recorded before and one hour after the nialamide injection were 95 mm Hg (S. E. M. 4.3, n = 4) and 96 mm Hg (S. E. M. 4.3, n = 4) (P > 0.1). However, after this pretreatment injection of the same dose of L-DOPA (25 mg/kg) caused a rapid increase in the mean arterial blood pressure. Following a maximal effect 15–20 min. after administration, the pressure increased significantly (P < 0.001) from 96 mm Hg (S. E. M. 4.3, n = 4) to 133 mm Hg (S. E. M. 4.3, n = 4). The duration of the increase was at least 60 minutes.

Discussion

Previous reports on the blood pressure effects of DA in the rat are scanty. However, a single dose of DA produced a pressor response in the anaesthetized rat (HORNYKIEWICZ & OBENAUS 1958). Hypertensive effects of equivalent amounts of injected DA were likewise found in the anaesthetized cat (HOLTZ & CREDNER 1942) or after intravenous infusion in conscious man (HORWITZ *et al.* 1962). However, other species may react with a decrease in blood pressure. Thus small doses (less than 3 µg/kg) elicit purely depressor responses in the anaesthetized dog (MACDONALD & GOLDBERG 1962; EBLE 1964) while larger doses result in a pressor response. Similar reactions are encountered in anaesthetized rabbits or guinea-pigs (HOLTZ & CREDNER 1942; HORNYKIEWICZ 1958; HORNYKIEWICZ & OBENAUS 1958).

Several explanations have been suggested for these variable effects of DA on the cardiovascular system (MACDONALD & GOLDBERG 1962; HORNYKIEWICZ 1958; HOLTZ & PALM 1966). In view of the varying species and tissues studied, it is probably difficult to arrive at a definite conclusion regarding the mechanism by which DA effects blood pressure. However, considering the poor penetration of this cathecholamine through the blood brain barrier (BERTLER *et al.* 1966), its actions must be preferentially of peripheral origin. In the present study at least two major principles may be considered, namely direct activation of adrenergic receptors or indirect effects via the sympathetic nerve endings. The present finding that PBZ blocked or considerably diminished the pressor response to DA clearly indicates an α-adrenergic effect. It is shown that infusion of DA in the rat, over a wide dose range, results in a pressor response. With regard to the shape of the infusion curve it

notable that the infusion of DA up to 4 μg/min. results in a relatively moderate hypertensive response but that higher doses produce a marked hypertensive reaction. Various explanations could be offered for this dose-effect relationship, e. g. an elimination mechanism for DA might become saturated in a dose range around 4 μg/min. This relationship needs further elucidation by the determination of plasma dopamine.

When DA was injected before and after blockade of the membrane pump mechanism by PTP, there was only an augmentation of the magnitude of pressor response to DA in two of the five doses tested. The duration of the hypertensive response seen after DA was not found to be enhanced after impairment of the membrane pump. Since blockade of this amine concentrating mechanism did not diminish the hypertensive reaction seen after DA, it seems less likely that the administered DA would have exerted its major action through an indirect effect via the sympathetic nerve endings. On the other hand, there was no clear-cut augmentation of magnitude and duration of the hypertensive response to DA like that seen after NA. Assuming that in these experiments NA acted through direct stimulation of adrenergic receptors only, this finding indicates that the effect seen after DA was not due to pure receptor stimulation. Further, the mixed picture with enhancement of hypertensive response to the higher doses of DA but not to the lower doses after membrane pump blockade, might support the view of a mixed effect with regard to direct and indirect mechanisms.

A combination of direct and indirect effects of DA has also been suggested for the cat nictitating membrane (TSAI *et al.* 1967), while in the human eye, mainly indirect actions were found (SPIERS & CALNE 1969). Here, too, species and organ differences may explain the variable results.

In view of the findings of SMITH (1966), TSAI *et al.* (1967) and OBIANWU (1969) indirectly acting amines should be potentiated after inhibition of the major intraneuronal monoamine inactivation mechanism. In the present investigation no such augmentation of the hypertensive response seen after DA was found after pretreatment with a potent inhibitor of monoamine oxidase. These data indicate a major directly acting mechanism of DA.

The mixed action of DA probably also applies to experiments in which its precursor L-DOPA produces a hypertensive effect in conscious rats (HENNING & RUBENSON 1970b). However, since the DA formed from exogenously administered L-DOPA accumulates largely intraneuronally, close to the NA storage sites (MALMFORS 1965; MUSACCHIO *et al.* 1966), it is possible that indirect actions of DA are mainly seen in such experiments. If this is so the pressor response should be augmented by an inhibitor of intraneuronal monoamine oxidase. The present data are in accordance with this assumption. After pretreatment with a potent inhibitor of intraneuronal monoamine oxidase, the administration of L-DOPA results in a rapid hyper-

tensive response, whereas the same dose of L-DOPA alone has no effect on the blood pressure. This has previously been shown for the action of L-DOPA on the blood pressure in anaesthetized cats (BALZER & HOLTZ 1956).

Acknowledgments

This work has been supported by grants from the Faculty of Medicine, University of Göteborg; the Swedish State Medical Research Council (No. B70-14X-155-06B). For generous gifts of drugs, I am obliged to Swedish Pfizer, Näsbypark (Nialamide), AB Leo, Hälsingborg, Sweden (phenoxybenzamine), Merck Sharp & Dohme International (protriptylin).

I am indepted to Miss Anna Jansson and Miss Ann-Sofi Järnström for skilful technical assistance.

REFERENCES

Balzer, H. & P. Holtz: Beeinflussung der Wirkung biogener Amine durch Hemmung der Aminooxydase. *Arch. exp. Path. Pharmakol.* 1956, **227**, 547–558.

Bertler, A., B. Falck, C. H. Owman & E. Rosengren: The localization of monoaminergic blood brain barrier mechanisms. *Pharm. Rev.* 1966, **18**, no. 1, 369–385.

Eble, J. N.: A proposed mechanism for the depressor effect of dopamine in the anaesthesized dog. *J. Pharmacol exp. Therap.* 1964, **145**, 64–70.

Fleckenstein, A. & J. H. Burn: The effect of denervation on the action of sympathomimetic amines on the nictating membrane. *Brit. J. Pharmacol.* 1953, **8**, 69–78.

Goldberg, L. I. & A. Sjoerdsma: Effects of several monoamine oxidase inhibitors on the cardiovascular action of naturally occurring amines in the dog. *J. Pharmacol. exp. Therap.* 1959, **127**, 212–218.

Henning, M.: Studies on the mode of action of α-methyldopa. *Acta physiol. scand.* 1969, suppl. 322.

Henning, M. & A. Rubenson: Evidence for a centrally mediated hypotensive effect of L-dopa in the rat. *J. Pharm. Pharmacol.* 1970a, **22**, 241–243.

Henning, M. & A. Rubenson: Central hypotensive effect of L-3,4-dihydroxyphenylalanine in the rat. *J. Pharm. Pharmacol.* 1970b, **22**, 553–560.

Holtz, P. & K. Credner: Die enzymatische Entstehung von Oxytyramin im Organismus und die physiologische Bedeutung der Dopadecarboxylase. *Arch. exp. Path. Pharmakol.* 1942/43, **200**, 356–388.

Holtz, P. & D. Palm: Brenzkatechinamine und andere sympathicomimetische Amine. *Ergebn. Physiol.* 1966, **58**, 1–580.

Hornykiewicz, O.: The action of dopamine on the arterial blood pressure of the guineapig. *Brit. J. Pharmacol* 1958, **13**, 91–94.

Hornykiewicz, O. & H. Obenaus: Beeinflussung der blutdrucksenkenden Wirkung von Dopamin und Adrenalin durch Lysergsäure-diäthylamid. *Wiener klin. Wschr.* 1958, **70**, 948–949.

Horwitz, D., S. M. Fox & L. I. Goldberg: Effects of dopamine in man. *Circulation Res.* 1962, **10**, 237–243.

MacDonald, R. H. & L. I. Goldberg: Analysis of the cardiovascular effects of dopamine in the dog (abstract). *Fed. Proc.* 1962, **21**, 127.

Malmfors, T.: Studies on adrenergic nerves. *Acta physiol. scand.* 1965, **64**, Suppl. 248, 1–93.

Maxwell, G. M., G. G. Rowe, C. A. Castillo, J. E. Clifford, S. Afonso & C. W. Crumpton: The effect of dopamine (3-hydroxytyramine) upon the systemic, pulmonary and cardiac haemodynamics and metabolism of intact dog. *Arch. int. Pharmacodyn.* 1960, **129**, 62–69.

Musacchio, J. M., J. E. Fischer & I. J. Kopin: Subcellular distribution and release by sympathetic nerve stimulation of dopamine and α-methyldopamine. *J. Pharmacol. exp. Therap.* 1966, **152**, 51–55.

Obianwu, H. O.: Possible functional differentiation between the stores from which adrenergic nerve stimulation, tyramine and amphetamine release noradrenaline *Acta physiol. scand.* 1969, **75**, 92–101.

Smith, C. B.: The role of monoamine oxidase in the intraneuronal metabolism of norepinephrine released by indirectly acting sympathomimetic amines or by adrenergic nerve stimulation. *J. Pharmacol. exp. Therap.* 1966, **151**, 207–220.

Spiers, A. S. D. & D. B. Calne: Action of dopamine on the human iris. *Brit. med. J.* 1969, **4**, 333–335.

Tsai, T. H., S. Z. Langer & U. Trendelenburg: Effects of dopamine and α-methyldopamine on smooth muscle and on the cardiac pacemaker. *J. Pharmacol. exp. Therap.* 1967, **156**, 310–324.

Acta pharmacol. et toxicol. 1971, 29, 145–154.

From the Department of Pharmacology, University of Göteborg,
Göteborg, Sweden

Effects of 5-Hydroxytryptophan on Arterial Blood Pressure, Body Temperature and Tissue Monoamines in the Rat

By

Matts Henning and Allan Rubenson

(Received December 1, 1970)

Abstract: The mean arterial blood pressure in conscious rats was recorded by means of in-dwelling arterial catheters. DL-5-hydroxytryptophan (5-HTP) 200 mg/kg intraperitoneally lowered the blood pressure significantly after 20 min. Following the administration of the peripheral decarboxylase inhibitor L-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid (MK 486) 100 mg/kg intraperitoneally, 5-HTP 200 mg/kg intraperitoneally caused only a slight lowering of the blood pressure after 1–2 hrs. 5-HTP 50 mg/kg given intraperitoneally after a combined pretreatment with a MAO inhibitor (nialamide) 100 mg/kg intraperitoneally and MK 486 100 mg/kg produced characteristic effects on gross behaviour after 20 min. No significant effects of 5-HTP on the blood pressure were observed in these experiments. 5-HTP 200 mg/kg intraperitoneally caused a hypothermia which was prevented by MK 486 100 mg/kg. After pretreatment with nialamide and MK 486 as in the blood pressure experiments, 5-HTP 50 mg/kg produced an initial hypothermia followed by a pronounced rise in body temperature. 5-HTP 200 mg/kg intraperitoneally resulted in a marked accumulation of 5-hydroxytryptamine (5-HT) in the heart and brain. Pretreatment with MK 486 100 mg/kg prevented the increase in heart 5-HT but did not change that in the brain Brain dopamine (DA) but not brain and heart noradrenaline (NA) decreased significantly. 5-HTP 50 mg/kg after the administration of nialamide and MK 486 increased the brain 5-HT but caused no change in heart 5-HT Nialamide and MK 486 per se increased brain DA and NA significantly; 5-HTP largely prevented the increase in brain DA but not in brain NA. It is concluded that 5-HTP has a hypothermic and a hypotensive action due to the effect of 5-HT on the extracerebral structures, while the hyperthermic effect may have a central nervous origin. 5-HT formed from 5-HTP lowers brain DA by displacement.

Key-words: Conscious rats – blood pressure – temperature – 5-hydroxytryptophan – dopa decarboxylase inhibition.

In recent years evidence has accumulated that 5-hydroxytryptamine (5-HT) may serve as a transmitter in the central nervous system. 5-HT-containing cell bodies have been demonstrated by fluorescence microscopy in the lower brain

stem in the rat. Further, 5-HT terminals have been demonstrated in this region as well as in other regions in the brain. In the spinal cord they are particularly abundant in the sympathetic lateral column (FUXE *et al.* 1965).

The function of these 5-HT neurons is incompletely understood. Administration of 5-hydroxytryptophan (5-HTP) which in contrast to 5-HT readily penetrates the blood brain barrier produces characteristic behavioural changes, particularly after inhibition of monoamine oxidase (MAO). The typical 5-HTP syndrome includes sedation, tremors in the head and front extremities as well as extension and abduction of the hind legs (GARATTINI & VALZELLI 1965; MANTEGAZZINI 1966). An interesting effect of 5-HTP is the inhibition of insulin-induced depletion of adrenaline from mouse adrenals (ANDÉN *et al.* 1964), possibly indicating that 5-HT acts as an inhibitory transmitter to the sympatho-adrenal preganglionic neurons in the spinal cord.

The present study was initiated in order to examine the possible role of central 5-HT mechanisms in the regulation of blood pressure, by studying the effects of 5-HTP. Previous investigations in this field are scanty and difficult to interpret since the peripheral effects of 5-HT formed from 5-HTP may obscure the picture; 5-HT may have qualitatively different effects on the blood pressure even in the same species (GARATTINI & VALZELLI 1965).

In recent investigations we have shown that it is possible to differentiate between central and peripheral components with regard to the actions of L-DOPA and DL-metatyrosine by the selective inhibition of the peripheral DOPA decarboxylase (HENNING & RUBENSON 1970a & b; RUBENSON 1970 unpublished results). Experiments were therefore set up to examine the influence of 5-HTP on the mean arterial blood pressure in conscious rats after pretreatment with the peripheral decarboxylase inhibitor L-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid. In some experiments the animals were also given the MAO inhibitor, nialamide. In correlative biochemical experiments tissue levels of 5-HT, noradrenaline (NA), and dopamine (DA) were examined.

Methods

Male Sprague Dawley rats weighing 200–250 g were used in all experiments. The mean arterial pressure was recorded by means of in-dwelling arterial catheters connected to Statham P23Dc pressure transducers connected with a Grass polygraph. The technical details have been described previously (HENNING 1969). The blood pressure values represent averages of recordings for ten minute periods, before and after the administration of drugs as given in "Results". The values taken 20 min. after 5-HTP are the average of the period 17,5–22,5 min. after 5-HTP. For doses of drugs and time intervals, see "Results". Tests of significance were performed by analysis of variance with two independent criteria of classification followed by t-test. For rectal temperature measurements a "Tele-thermometer" (Yellow Springs Instrument Co. Inc., Ohio, USA)

was used. The room temperature was 24°. Basal temperature values were obtained by 3 measurements during a 15 min. period. Drug effects were expressed as deviations from the mean of these values.

In biochemical studies organs from two animals were pooled. 5-HT was determined according to ANDÉN & MAGNUSSON (1967). DA and NA were measured as described by CARLSSSON & LINDQVIST (1962), and BERTLER *et al.* (1958), respectively. The values were treated statistically by analysis of variance and t-test.

DL-5-hydroxytryptophan and nialamide were dissolved in saline with a few drops of N-HCL (pH adjusted to 6). L-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid (MK 486) was dissolved in saline. In both blood pressure and biochemical experiments care was taken to prevent hypothermia.

Results

Blood pressure experiments.

5-HTP series. 5-HTP was given intraperitoneally in various doses from 200 mg/kg up to 800 mg/kg. Clear cut behavioural changes were only observed after 800 mg/kg. These animals rapidly developed tremors of the head and fore-limbs and extension and abduction of the hind legs and often died within 0.5–2 hrs.

Fig. 1 shows the changes in the mean arterial blood pressure after 5-HTP (200 mg/kg). There was a significant fall in pressure after 20 min. with a return to control values after 1 and 2 hrs ($P < 0.005$).

MK 486 and 5-HTP series. After pretreatment with MK 486 (100 mg/kg) the injection of 5-HTP 200 mg/kg had only small effects on the mean arterial blood pressure. Apart from a slight apparent sedation no definite gross behavioural changes were observed. During the first hour after administration no significant changes occurred, but 1 hr and 2 hrs after 5-HTP there was a slight lowering of the blood pressure ($P < 0.05$ as compared to the value before 5-HTP; $P > 0.1$ when compared to the value before MK 486). The blood pressure values taken 3 and 6 hrs after the 5-HTP injection were not significantly different from the control values ($P > 0.1$).

Nialamide + MK 486 + 5-HTP series. The injection of 5-HTP 50 mg/kg 60 min. after nialamide 100 mg/kg and 30 min. after MK 486 100 mg/kg resulted in a pronounced 5-HTP syndrome starting 20 min. after the injection of 5-HTP and reaching a maximum after 50–60 min. Thirty min. after nialamide there was a significant fall in blood pressure ($P < 0.005$). However, no further significant changes were observed in the blood pressure values taken after MK 486 or up to 1 hr after 5-HTP ($P > 0.1$). As a rule these animals deteriorated and died 2–3 hrs after 5-HTP.

Control experiments were performed with nialamide 100 mg/kg and MK

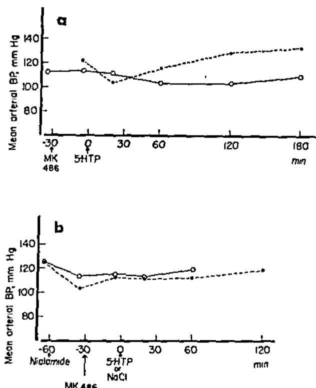


Fig. 1. Changes in mean arterial blood pressure of conscious rats after intraperitoneal injections of drugs as indicated. Error variance was calculated by analysis of variance with two independent criteria of classification (treatment vs. individuals).
a. *5-hydroxytryptophan (5-HTP) 200 mg/kg alone (broken line; error variance 102.3, n = 7) and 30 min. after MK 486 100 mg/kg (solid line; error variance 50.8, n = 6)*
b. 5-hydroxytryptophan (5-HTP) 50 mg/kg 30 min. after MK 486 100 mg/kg and 60 min after nialamide 100 mg/kg (solid line; error variance 44.3, n = 6). Broken line indicates controls given 0.9 % NaCl 5 ml/kg 30 min. after MK 486 100 mg/kg and 60 min. after nialamide 100 mg/kg (error variance 67.6, n = 6).

486 100 mg/kg 60 and 30 min. before saline, respectively. A significant decrease in blood pressure was again observed 30 min. after nialamide 100 mg/kg. The blood pressure remained significantly decreased up to 1 hr after the administration of MK 486.

Temperature measurement.

In a separate series of experiments the rectal temperature was recorded. The results are shown in fig. 2. 5-HTP 200 mg/kg alone caused a rapid and long-lasting lowering of rectal temperature. The same dose of 5-HTP 30 min. after MK 486 (100 mg/kg) also lowered the body temperature. However, this fall in temperature was less pronounced than after 5-HTP only. After pre-

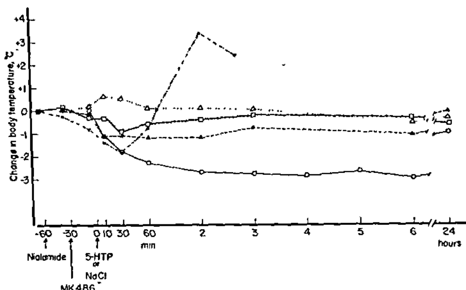


Fig. 2. Changes in rectal temperature of rats treated intraperitoneally as follows: 5-hydroxytryptophan (5-HTP) 200 mg/kg (open circles); error variance 0.159; 5-HTP 200 mg/kg 30 min. after MK 486 100 mg/kg (filled triangles); error variance 0.144; 5-HTP 50 mg/kg 30 min. after MK 486 100 mg/kg and 60 min. after nialamide 100 mg/kg (solid circles); error variance 1.54; 0.9 % NaCl 5 ml/kg 30 min. after MK 486 100 mg/kg (open triangles), error variance 0.050; 0.9 % NaCl 5 ml/kg 30 min. after MK 486 100 mg/kg and 60 min. after nialamide 100 mg/kg (open squares); error variance 0.140.

Each drug combination was given to 3 animals. The values are means of the deviation from the basal temperature. Error variance was calculated by analysis of variance. Difference between temperature deviations (analysis of variance and t-test):
5-HTP ⟷ MK 486 + 5-HTP after 2 hrs: $P < 0.01$.
Nialamide + MK 486 + saline ⟷ nialamide + MK 486 + 5-HTP after 30 min. and 2 hrs: $P < 0.05$ (values after 60 and 70′ excluded in analysis of variance due to variable time of onset of hyperthermia. Error variance = 0.094), and $P < 0.001$, respectively.

treatment with nialamide 100 mg/kg and MK 486 100 mg/kg (60 and 30 min. previously), 5-HTP 50 mg/kg produced a biphasic temperature response with an initial hypothermia lasting about 1 hr followed by a rise in temperature which persisted until the animals deteriorated and died after 3–6 hrs. In control experiments MK 486 (100 mg/kg) alone and in combination with nialamide 100 mg/kg had a slight hypothermic effect.

Biochemical experiments.

All the drugs were given in doses and at time intervals similar to those in the blood pressure experiments. The animals were sacrificed 1 hr after 5-HTP or saline.

Table 1.

Tissue levels of noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) after injection of 5-hydroxytryptophan (5-HTP) or saline following different pretreatments. The animals were killed 1 hr after 5-HTP or saline injection. All drugs were given intraperitoneally. Values are means in μg/g tissue and the number of experiments is given in brackets. n. s. = not significant.

	Brain			Heart	
	5-HT	NA	DA	5-HT	NA
A. Untreated	0.30 (4)	0.41 (3)	0.74 (4)	0.34 (3)	0.98 (4)
B. 5-HTP 200 mg/kg	1.41 (3)	0.36 (3)	0.67 (3)	2.81 (3)	0.85 (3)
C. 0.9 % NaCl 5 ml/kg 30 min. after MK 486 100 mg/kg	0.40 (4)	0.37 (4)	0.72 (4)	0.35 (4)	0.92 (4)
D. 5-HTP 200 mg/kg 30 min. after MK 486 100 mg/kg	1.49 (5)	0.35 (5)	0.57 (5)	0.54 (5)	0.88 (5)
E. 0.9 % saline 5 ml/kg 30 min. after MK 486 100 mg/kg and 60 min. after nialamide 100 mg/kg	0.59 (3)	0.58 (3)	1.26 (3)	0.25 (3)	1.33 (3)
F. 5-HTP 50 mg/kg 30 min. after MK 486 100 mg/kg and 60 min. after nialamide 100 mg/kg	1.63 (3)	0.52 (3)	0.82 (3)	0.41 (3)	1.00 (3)
Variance within group	0.0149	0.0016	0.0060	0.0126	0.0348
A – B	< 0.001	n. s.	n. s.	< 0.001	n. s.
A – D	n. s.	n. s.	n. s.	n. s.	n. s.
B – D	n. s.	n. s.	n. s.	< 0.001	n. s.
C – D	< 0.001	n. s.	< 0.025	< 0.025	n. s.
C – E	n. s.	< 0.001	< 0.001	n. s.	n. s.
E – F	< 0.001	n. s.	< 0.001	n. s.	n. s.

5-HTP series. Injection of 5-HTP (200 mg/kg) alone resulted in a marked accumulation of 5-HT in the heart and brain. No significant changes occurred in the heart NA and brain DA and NA as compared to the untreated controls ($P > 0.1$, table 1).

MK 486 + 5-HTP series. After pretreatment with MK 486 (100 mg/kg) only small amounts of 5-HT were found in the heart. The accumulation of 5-HT in the brain did not differ from that seen after 5-HTP alone ($P > 0.1$). No significant changes occurred in the heart and brain NA. However, there was a significant decrease in brain DA as compared to the controls (untreated or treated with MK 486; $P < 0.025$).

Nialamide + MK 486 + 5-HTP series. The injection of 5-HTP (50 mg/kg) after pretreatment with nialamide (100 mg/kg) and MK 486 (100 mg/kg) produced no significant change in heart 5-HT ($P > 0.1$) as compared to the untreated or controls treated with nialamide and MK 486. However, in the brain there was a highly significant increase in 5-HT as compared to the same type of controls.

No significant changes occurred in the heart NA level. There was no change in brain NA levels as compared to the controls treated with nialamide + MK 486. (However, the latter pretreatment alone increased the brain NA content significantly ($P < 0.001$).

Pretreatment with nialamide + MK 486 resulted a pronounced increase in brain DA ($P < 0.001$). However, the injection of 5-HTP (50 mg/kg) following the same pretreatment gave a significant decrease in brain DA content ($P < 0.001$).

Discussion

Administration of 5-HTP alone resulted in a significant lowering of the mean arterial blood pressure. Pretreatment with the peripheral decarboxylase inhibitor MK 486 largely prevented this effect. These findings suggest that the hypotensive effect of 5-HTP alone is preferentially of peripheral origin and is mediated via its decarboxylation product, 5-HT. The small decrease in blood pressure seen 1 and 2 hrs after MK 486 + 5-HTP may have been caused by the peripheral actions of 5-HT formed from 5-HTP because of insufficient decarboxylase inhibition by MK 486; the biochemical experiments showed a small but significant increase in heart 5-HT levels as compared to MK 486 only. A possible central nervous source of the slight hypotensive effect of 5-HTP after peripheral decarboxylase inhibition cannot be excluded, but appears less likely in view of the results obtained with MK 486 + 5-HTP after MAO inhibition. Thus, the administration of 5-HTP in a dose producing clear cut behavioural changes in animals pretreated with nialamide and MK 486, failed to affect the blood pressure significantly. In these experiments brain 5-HT levels were at least as high as after MK 486 + 5-HTP.

Few previous studies on the effects of 5-HTP on blood pressure are available; the results are contradictory. Moderate doses of 5-HTP have been reported to lower blood pressure in anaesthetized dogs (CROHNHEIM & GOURZIS 1960) or cats (ERSPAMER *et al.* 1960). On the other hand SANGHVI & GERSHON (1970) found a hypertensive effect of 5-HTP in the conscious dog. However, in these investigations it has not been possible to differentiate central and peripheral effects of 5-HT formed in the tissues.

The temperature recordings revealed that 5-HTP produces hypothermia in rats. This is in contrast to other species in which mainly hyperthermic

reactions have been found (GARATTINI & VALZELLI 1965). Inhibition of peripheral dopa decarboxylase weakened the hypothermic effect of 5-HTP, possibly indicating an extracerebral component involved in the lowering of body temperature caused by 5-HTP. In animals pretreated with a MAO inhibitor in combination with MK 486, the administration of 5-HTP resulted in a biphasic temperature response; initially there was hypothermia followed by a pronounced hyperthermic reaction. A potentiation of the pyretogenic effect of 5-HTP by MAO inhibition in rabbits has previously been shown by HORITA & GOGERTY (1958). Furthermore it can be shown that the hyperthermic effect of 5-HTP in rabbits is potentiated after inhibition of peripheral dopa decarboxylase (HORITA & HAMILTON 1970). Taken together these findings may indicate a central nervous origin of the hyperthermic effect of 5-HTP.

It is well established that 5-HTP rapidly is decarboxylated to 5-HT in most tissues (UDENFRIEND *et al.* 1957a & b; BOGDANSKI *et al.* 1958). 5-HTP is probably decarboxylated by the same decarboxylase as dihydroxyphenylalanine (DOPA) (ROSENGREN 1960; SOURKES 1966; GARATTINI & VALZELLI 1965). The present experiments confirm that the administration of 5-HTP causes a marked accumulation of 5-HT in the heart and brain as previously shown by several investigators (GARATTINI & VALZELLI 1965). The peripheral DOPA decarboxylase inhibitor MK 486 (PORTER *et al.* 1962) largely prevented the formation of 5-HT in the heart. This might be expected to result in an increased availability of 5-HTP to the brain; however, there was no change in the accumulation of 5-HT in the brain after pretreatment with MK 486. Possibly, an increased 5-HT formation in the brain parenchyma was balanced by a decreased intra-capillary 5-HT formation due to the inhibition of extra-neuronal decarboxylase (CONSTANTINIDIS *et al.* 1969). Similar observations were made with regard to metatyramine after the injection of DL-metatyrosine (RUBENSON 1970 unpublished results). Thus, MK 486 appears to inhibit the peripheral decarboxylation of these amino acids but does not result in a net increase of the central formation of the amino acid metabolites (PORTER *et al.* 1962). In the present experiments no changes in brain and heart NA content were found after the administration of 5-HTP. However, there was a fall in brain DA after MK 486 plus 5-HTP. Furthermore, this effect was more pronounced after MAO inhibition. This might indicate a displacement of DA by 5-HT.

Thus the administration of 5-HTP, results in a hypotensive action, probably mediated via 5-HT acting on the extracerebral structures. When the decarboxylation of 5-HTP is largely prevented in peripheral tissues, it is not possible to demonstrate any blood pressure effects of 5-HTP which can be attributed to central nervous actions. At the same time, there is no significant change in brain levels of NA but a decrease in brain DA content. In this

connection it is interesting to note that L-DOPA and m-tyrosine lower the mean arterial blood pressure in the conscious rat after peripheral decarboxylase inhibition and that this action may be of central noradrenergic nature (HENNING & RUBENSON 1970a & b; RUBENSON 1970 unpublished results). Furthermore, α-methyldopa may lower the blood pressure in doses which do not alter brain 5-HT levels (HENNING & MÅRILD, unpublished observation). However, it shold be emphasized that these results do not exclude the possibility that 5-HT mechanisms play a significant role in the regulation of blood pressure mechanisms.

Acknowledgements

The work has been supported by grants from the Faculty of Medicine, University of Göteborg, Göteborg and the Swedish State Medical Research Council (Nos. B70-14X-2863-02 and B70-14X-155-06B). For a generous gift of MK 486 we are obliged to Dr. C. A. Stone, Merck Sharp & Dohme. We are greatly indebted to Miss Anna Janson and Miss Ann-Sofie Järnström for skilful technical assistance.

REFERENCES

Andén, N.-E., A. Carlsson & N.-Å. Hillarp: Inhibition by 5-hydroxytryptophan of insulin-induced adrenaline depletion. *Acta pharmacol. et toxicol.* 1964, **21**, 183–186.

Andén, N.-E. & T. Magnusson: An improved method for the fluorimetric determination of 5-hydroxytryptamine in tissues. *Acta physiol. scand.* 1967, **69**, 87–94.

Bertler, A., A. Carlsson & E. Rosengren: A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958, **44**, 273–292.

Bogdanski, D. F, H. Weissbach & S. Udenfriend: Pharmacological studies with the serotonin precursor, 5-hydroxytryptophan. *J. Pharmacol. exp. Therap.* 1958, **122**, 182–194.

Carlsson, A. & M. Lindqvist: *In vivo* decarboxylation of α-methyl DOPA and α-methyl meta-tyrosine. *Acta physiol. scand.* 1962, **54**, 87–94.

Constantinidis, J., J. C. de la Torre, R. Tissot & F. Geissbuhler: La barrière capillaire pour la dopa dans le cerveau et les différents organes. *Psychopharmacologia (Berl.)* 1969, **15**, 75–87.

Crohnheim, G E. & J. T. Gourzis: Cardiovascular and behavioral effects of serotonin and related substances in dogs with and without reserpine premedication. *J. Pharmacol. exp. Therap.* 1960, **130**, 444–449.

Erspamer, V., A. Glässer & P. Mantegazzini: Pharmacological actions of 4-hydroxytryptamine and 4-hydroxytryptophan. *Experientia* 1960, **16**, 505–506.

Fuxe, K., A. Dahlstrom & N.-A. Hillarp: Central monoamine neurons and monoamine neuro-transmission *Excerpta Medica International Congress Series.* 1965, **87**, 419–434.

Garattini, S. & L. Valzelli: *Serotonin.* Amsterdam, Elsevier Publishing Company, 1965.

Henning, M.: *Studies on the mode of action of α-methyldopa. Acta physiol. scand.* 1969, suppl. 322.

Henning, M. & A. Rubenson: Evidence for a centrally mediated hypotensive effect of L-dopa in the rat. *J. Pharm. Pharmacol.* 1970a, 22, 241–243.

Henning, M. & A. Rubenson: Central hypotensive effect of L-3,4-dihydroxyphenylalanine in the rat. *J. Pharm. Pharmacol.* 1970b, 22, 553–560.

Horita, A. & J. H. Gogerty: The pyretogenic effect of 5-hydroxytryptophan and its comparison with that of LSD. *J. Pharmacol. exp. Therap.* 1958, 122, 195–200.

Horita, A. & A. E. Hamilton: Potentiation of the central actions of 5-hydroxytryptophan *in rabbits by DL-α-hydrazino-α-methyldopa. J. Pharm. Pharmacol. 1970,* 22, 389–391.

Mantegazzini, P.: In: Handbuch der experimentellen Pharmakologie. Vol. XIX. Eds.: O. Eichler & A. Farah, Springer Verlag, Berlin-Heidelberg-New York, 1966, 424–470.

Porter, C. C., L. S. Watson, D. C. Titus, J. A. Totaro & S. S. Byer: Inhibition of DOPA decarboxylase by the hydrazino analogue of α-methyl-DOPA. *Biochem. Pharmacol.* 1962, 11, 1067–1077.

Rosengren, E.: Are dihydroxyphenylalanine decarboxylase and 5-hydroxytryptophan *decarboxylase individual enzymes? Acta physiol. scand. 1960,* 49, *364–369.*

Sanghvi, I. & S. Gershon: Similarities between behavioral and pharmacological actions of yohimbine and 5-hydroxytryptophan in the conscious dog. *Eur J. Pharmac.* 1970, 11, 125–129.

Sourkes, T. L.: Dopa decarboxylase: substrates, coenzyme, inhibitors. *Pharmac. Rev.* 1966, 18, 53–60.

Udenfriend, S., H. Weissbach & D. F. Bogdanski: Biochemical findings relating to the action of serotonin. *Ann. N. Y. Acad. Sci.* 1957a, 66, 602–608.

Udenfriend, S., H. Weissbach & D. F. Bogdanski: Increase in tissue serotonin following *administration of its precursor 5-hydroxytryptophan. J. biol. Chem.* 1957b, 224, 803–810.

Acta pharmacol. et toxicol. 1971, 29, 155–163.

From AB Bofors, Nobel-Pharma, Mölndal, Sweden

Hydrolysis of Salicylsalicylic Acid in Human Blood and Plasma: A Comparison with Acetylsalicylic Acid

By

Lennart Harthon and Margit Hedström

(Received July 22, 1970)

Abstract: The hydrolysis *in vitro* of salicylsalicylic acid and acetylsalicylic acid was investigated in human plasma and whole blood. The rate of hydrolysis in plasma as well as in whole blood was considerably greater for acetylsalicylic acid than for salicylsalicylic acid. Acetylsalicylic acid was hydrolyzed much more rapidly in whole blood than in plasma while salicylsalicylic acid tended to hydrolyze more slowly in whole blood than in plasma. These differences may in part explain the differences in biological half-life between the two drugs.

Key-words: Salicylsalicylic acid – acetylsalicylic acid – hydrolysis – blood.

The spontaneous hydrolysis of acetylsalicylic acid (ASA) in aqueous solutions is well known. This reaction in blood and plasma is catalyzed by ester cleaving enzymes (LESTER *et al.* 1946). At a low concentration of substrate and a constant concentration of the enzyme the reaction though of the second order can be treated mathematically as being of the first order.

The enzyme hydrolyzing acetylsalicylic acid is often called acetylsalicylic acid esterase (ASA-esterase) but is probably not a single enzyme but consists of a group of arylesterases. In human whole blood the hydrolysis of ASA is about twice as fast as in human plasma. The reaction in whole blood can also be treated as being of first order (HARRIS & RIEGELMAN 1967). The enzymes in human serum which hydrolyze acetylsalicylic acid are partly inhibited by physostigmine (MORGAN & TRUITT 1965) and are inactivated by heat (SMITH 1960). The hydrolysis of the chemically related phenylacetate by haemolyzed human erythrocytes is inhibited to about 50 per cent by 10^{-5} M physostigmine (VINCENT 1967). The optimum pH for the hydrolysis of acetylsalicylic acid with an enzyme preparation from dog liver is between 6.0 and 6.5 (SMITH 1960).

As far as we know the hydrolysis of salicylsalicylic acid (SSA) in blood or plasma has not been investigated. The effects of pancreatine and

SSA *in vitro* were, however, studied by HANZLIK & PRESHO (1926). In humans they found that after ingestion of salicylsalicylic acid, 10 per cent of the total amount of salicylates excreted consisted of unchanged salicylsalicylic acid. On the basis of their experiments they therefore concluded that in tissues the substance is largely split into two molecules of salicylic acid. In patients treated with the drug NORDQVIST (1964) has demonstrated that unhydrolyzed salicylsalicylic acid amounted to 30–40 per cent of the total concentration of salicylates present in the plasma.

The purpose of this study was to compare the hydrolysis *in vitro* of salicylsalicylic acid in human plasma with that of acetylsalicylic acid. We also investigated whether in the case of salicylsalicylic acid, whole blood has a greater hydrolyzing activity than plasma.

Material and Methods

Blood with heparin added (5 i. u./ml) was obtained under sterile conditions from five healthy blood-donors. The plasma was prepared by centrifugation, carefully avoiding haemolysis. In all the experiments in which the activity of whole blood was compared with that of plasma the same blood was used. Preliminary experiments showed that the blood could be stored for about a week in a refrigator at 5–6° without any obvious loss of hydrolyzing activity. Due to the low solubility of salicylsalicylic acid (less than 50 mg/l in water and 0.1 M hydrochloric acid) the substances were added dissolved in ethanol. The concentration of ethanol was 5 per cent during all incubations All the experiments were performed at 37° in a water bath in an atmosphere of carbogen (94 % O_2 – 6 % CO_2) to maintain a pH of 7.4 Hydrolysis in buffer was carried out in Krebs-Ringer bicarbonate buffer at pH 7.4 under otherwise identical conditions.

A pharmacotherapeutic active concentration of the drugs was chosen It was approximately 10 mg per 100 ml (ASA 5.6×10^{-4} mol/l; SSA 3.9×10^{-4} mol/l) in all the experiments. Calculations were, however, carried out on the basis of equivalent amounts of salicylic acid.

Salicylic acid liberated by hydrolysis was determined according to the method of BRODIE *et al.* (1944) and it was verified that this method was also applicable in the presence of salicylsalicylic acid The total amount of salicylic acid was determined after previous alkaline hydrolysis. The zero values of free salicylic acid were estimated immediately after the substrate was added and adequate mixing had been performed When ever possible the hydrolysis was then followed for 4–6 hours and samples drawn every half hour for two hours and thereafter every hour.

The experiments in plasma were performed simultaneously on plasma from the same donor In buffer the experiments were also performed in parallel.

When comparing hydrolysis in plasma and in whole blood one of the substances was investigated in the two media at the same time

Results

Influence of ethanol. It could be assumed that the ethanol present during incubation would reduce the velocity of hydrolysis. Experiments with different concentrations of ethanol and extrapolation of the rates to zero

concentration, showed that 5 per cent of ethanol reduced the velocity of hydrolysis by 11±3 per cent, a figure which is applicable for ASA as well as for SSA. In the calculation of the results no corrections have been made for this inhibition by alcohol.

Spontaneous hydrolysis in buffer. Two experiments with each substance were performed to estimate the hydrolysis due to buffer. In each experiment the amount of salicylic acid formed was determined at 7–8 different times for at least four hours. Correlation for linear regression of the logarithms of remaining substrate against time, according to the method of least square was made on a computer to obtain constants of first order kinetics. The means of the slopes of these lines were for ASA 0.0181 and for SSA 0.0102. The correlation coefficients ranged from 0.987 to 0.996 showing a high degree of correlation and the standard deviation of y about the regression line varied between 0.0024 and 0.0040. A paired comparison of the regression coefficients (BROWNLEE 1960) for lines representing the two substances shows that they differ significantly ($P < 0.001$) in both experiments. This means that the hydrolysis in buffer at pH 7.4 of salicylsalicylic acid is significantly slower than the hydrolysis of acetylsalicylic acid (fig. 1). Taking the means of the slopes (b) of the regression lines for the two experiments on each substance the velocity constants $K_1 = b \cdot 2.303$ for a first order reaction could be calculated to 0.0405 and 0.0235 hr^{-1} for acetylsalicylic acid and

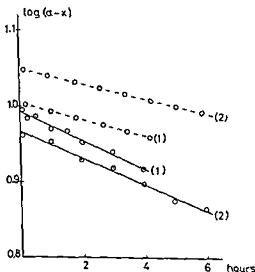


Fig 1. Hydrolysis in buffer at pH 7.4 of salicylsalicylic acid, O-- O, and acetylsalicylic acid O——O. Logarithm of remaining substrate against time. Circles represent experimental points and the lines represent the calculated regression lines. Figures in brackets refer to the different experiments.

salicylsalicylic acid respectively. An earlier estimation of K_1 for acetylsalicylic acid was made in citrate buffer by EDWARDS (1952). His value for $K_1 = 0.0383\ hr^{-1}$ is in good agreement with our own results.

Hydrolysis in human plasma. Three experiments were performed to investigate the rate of hydrolysis. Graphically the results are presented in fig. 2. The best equations satisfying the experimental data obtained in the three plasma were: for ASA $y = -0.0859\ x + 1.062$ (A); $y = -0.133\ x + 0.977$ (B); $y = -0.187\ x + 0.957$ (C) and for SSA $y = -0.0272\ x + 0.996$ (A); $y = -0.0263\ x + 1.050$ (B); $y = -0.0325\ x + 1.016$ (C).

The correlation coefficients were estimated to 0.994–0.998 in the different experiments.

The velocity constant K_1 for the hydrolysis of acetylsalicylic acid in plasma was calculated to be 0.198; 0.306 and 0.431 hr^{-1} and for salicylsalicylic acid

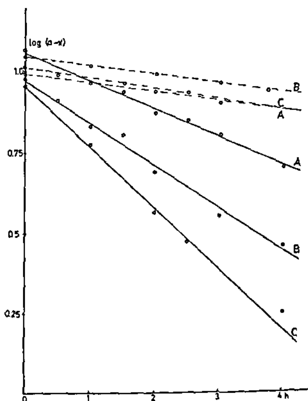


Fig. 2. Plasma hydrolysis of salicylsalicylic acid (O – – O) and acetylsalicylic acid (O——O) in human blood plasma. All ten experimental points are not shown in the figures. The letters refer to the plasma samples and correspond to those in the text.

0.0626; 0.0606 and 0.0748 hr^{-1}. The corresponding time for 50 per cent hydrolysis was 3.5; and 1.6 hr for ASA and 11.1; 11.4 and 9.3 hrs for SSA. In these figures no corrections have been made for the spontaneous buffer hydrolysis. The figures are also included in table 2.

The statistical comparison of the regression coefficients made it clear that the hydrolysis of salicylsalicylic acid in each plasma sample was significantly slower than that of acetylsalicylic acid. It was also evident that the different plasma samples possessed different capacities for hydrolyzing acetylsalicylic acid.

If the figures obtained from plasma hydrolysis are corrected for the spontaneous buffer hydrolysis it is still evident that with a high degree of statistical significance ($P < 0.001$) acetylsalicylic acid is hydrolyzed more rapidly than salicylsalicylic acid.

Comparison of hydrolysis in whole blood and plasma. In our experiments we compared the hydrolysis of both substances in the blood and plasma from the same donor. Hydrolysis in the blood and plasma was investigated on the same occasion but there could be a delay of one or two days between the experiments with the two substances. Because of this delay a noticable loss in enzymatic activity of the blood or plasma could not be detected. With a highly significant correlation to a straight line ($r = 0.981 - 0.998$), the data obtained indicate that the hydrolysis in whole blood can also be treated as a reaction of the first order. In table 1 the coefficients for the straight lines computed from the experimental data are given in the same way as previously described.

Table 1.

Coefficients of regression lines $y = b\,x + 1$ for the hydrolysis of acetylsalicylic acid and salicylsalicylic acid in human plasma and blood. σy = standard deviation of y about the regression line, n = number of observations in the experiment.

Blood from donor	Plasma				Blood			
	− b	1	σy	n	− b	1	σy	n
			Acetylsalicylic acid					
C	0.204	0.992	0.0390	6	0.378	0.945	0.0427	7
D	0.185	0.920	0.0114	5	0.393	0.881	0.0251	5
E	0.159	0.880	0.0420	5	0.342	0.901	0.0288	8
			Salicylsalicylic acid					
C	0.0313	1.025	0.0107	5	0.0268	1.035	0.0061	7
D	0.0263	1.072	0.0045	5	0.0197	1.058	0.0049	9
E	0.0275	1.012	0.0084	6	0.0186	0.966	0.0025	10

Table 2.

Rate constants (K_1) and half-lives ($t_{1/2}$) for hydrolysis of acetylsalicylic acid and salicylsalicylic acid in human plasma and whole blood.

Blood from donor	Acetylsalicylic acid				Salicylsalicylic acid			
	Plasma		Whole Blood		Plasma		Whole Blood	
	K_1 (hr^{-1})	$t_{1/2}$ (hr)	K_1 (hr^{-1})	$t_{1/2}$ (hr)	K_1 (hr^{-1})	$t_{1/2}$ (hr)	K_1 (hr^{-1})	$t_{1/2}$ (hr)
A	0.198	3.50	–	–	0.0626	11.1	–	–
B	0.306	2.27	–	–	0.0606	11.4	–	–
C	0.431	1.61	–	–	0.0748	9.27	–	–
C	0.469	1.48	0.871	0.80	0.0721	9.61	0.0617	11.2
D	0.426	1.63	0.905	0.77	0.0606	11.4	0.0454	15.3
E	0.366	1.89	0.788	0.88	0.0633	11.0	0.0428	16.2

The hydrolysis curves obtained from the coefficients in table 1 are shown in fig. 3 so that the differences in the rate of hydrolysis of acetylsalicylic acid and salicylsalicylic acid in whole blood and in plasma can be seen. No experimental points have been plotted in order to get a clearer picture. It is evident from the figure that salicylsalicylic acid is hydrolyzed much more slowly than acetylsalicylic acid both in whole blood and in plasma.

Contrary to acetylsalicylic acid which is hydrolyzed much more rapidly in whole blood than in plasma ($P < 0.001$) the figure gives an indication that salicylsalicylic acid may be hydrolyzed more slowly in whole blood than in plasma. Statistical comparison of the regression coefficients from table 1 reveals that for salicylsalicylic acid there was no difference between the velocity of hydrolysis in whole blood or in plasma in blood C. In blood D and E, however, the hydrolysis proceeded more slowly in whole blood than in plasma ($P < 0.01$ and $P < 0.001$ respectively).

Similar discrepancies have also been observed in the hydrolysis of esters of nicotinic acid with human as well as with animal blood (unpublished results). An explanation of these divergences cannot be given but genetical differences may be involved.

From the coefficients for the hydrolysis curves the velocity constants and the half-lives (time for 50 per cent hydrolysis) were calculated. These results are given in table 2.

In a few experiments we compared the hydrolysis in a suspension of washed erythrocytes in buffer with the hydrolysis under the same conditions with haemolyzed cells without removal of the cell membranes. In the case of salicylsalicylic acid no difference in the rate of hydrolysis was observed

but acetylsalicylic acid was hydrolyzed slightly more rapidly in haemolyzed than in intact cells, the rate constants estimated being 12 and 16 per cent higher in two experiments. These differences were significant but of low order of probability ($P < 0.05$).

The whole blood used may have been partly haemolyzed due to the addition of heparin. It is not likely, however, that this influenced the results to any appreciable extent.

Discussion

From the experiments with hydrolysis in buffer at pH 7.4 it is evident that the ester binding of salicylsalicylic acid is more resistant than that of acetylsalicylic acid under the conditions used.

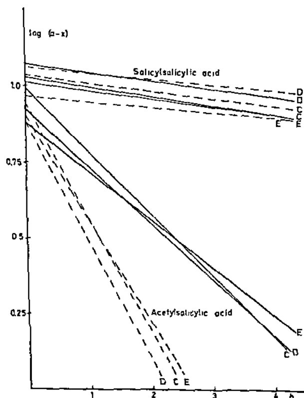


Fig. 3. Curves of hydrolysis of salicylsalicylic acid and acetylsalicylic acid constructed from the constants in table 1. --- hydrolysis in human whole blood and — hydrolysis in human plasma. The letters for each line refer to the corresponding table 1.

There is also a greater stability against ester cleaving enzymes in human plasma of the link between two esterified molecules of salicylic acid than if an acetyl group is linked to the phenolic group of salicylic acid.

The difference in the capacity of whole blood and plasma or serum to hydrolyze acetylsalicylic acid is well confirmed by the investigations of HARRIS & RIEGELMAN (1967) and also by previous authors (see MORGAN & TRUITT 1965 for references). This higher activity of whole blood may be explained by the findings of VINCENT (1967) who demonstrated that the cell stroma contains a cholinesterase sensitive to physostigmine and which is very active in hydrolyzing phenylacetate.

In one experiment salicylsalicylic acid was not significant but in two experiments it was significantly slower hydrolyzed in whole blood than in the corresponding plasma. Acetylsalicylic acid in all the experiments was hydrolyzed much more rapidly in whole blood. Experiments with isolated cells demonstrated an activity mainly located to the cell stroma. This cell bound enzyme also has a considerably higher capacity to hydrolyze acetylsalicylic acid.

In one experiment salicylsalicylic acid was not significant but in two experiments it was significantly slower hydrolyzed in whole blood than in the the salicylate blood levels in humans after an oral dose of these drugs and the kinetic constants of absorption and elimination calculated according to WIEGAND & SANDERS (1964). The biological half-life of acetylsalicylic acid was calculated to be 4.7 and for salicylsalicylic acid to be 7.8 hrs. The differences in the rate of hydrolysis found *in vitro* may at least partly contribute to an explanation of the differences found in biological half-lives.

REFERENCES

Brodie, B. B., S. Udenfriend & A. F. Coburn: The determination of salicylic acid in plasma. *J. Pharmacol. Exp. Therap.* 1944, 80, 114–117.

Brownlee, K. A.: *Industrial experimentation.* Her Majesty's Stationary Office, London 1960.

Edwards, L. J.: The hydrolysis of aspirin in guinea-pig plasma. *J. chem Soc.* 1952, 4114–4117.

Hanzlik, P. J. & N. E. Presho: Liberation of salicyl from and excretion of salicylsalicylate. *J. Pharmacol. Exp. Therap.* 1926, 26, 61–70.

Harris, P. A. & S. Riegelman: Acetylsalicylic acid hydrolysis in human blood and plasma. *J. Pharm. Sci* 1967, 56, 713–716.

Lester, D., G. Lolli & G A. Greenberg: The fate of acetylsalicylic acid. *J. Pharmacol. Exp. Therap.* 1946, 87, 329–342.

Morgan, A. M. & E. B. Truitt: Evaluation of acetylsalicylic acid esterase in aspirine metabolism. *J. Pharm. Sci.* 1965, 54, 1640–1646.

Nordqvist, P.: Lecture. Svenska Läkarsällskapets Riksstämma. Stockholm 1964.

Nordqvist, P., J. G. L. Harthon & R. Karlsson: Omsättningskinetik för salicylsalicylsyra, acetylsalicylsyra och natriumsalicylat hos människa. *Nord. Med.* 1965, 74, 1074–1076.

Smith, P. K.: The pharmacology of salicylates and related compounds. *Ann. N. Y. Acad. Sci.* 1960, **86**, 38–63.

Vincent, D.: L'arylestérase érythrocytaire principe et technique de son dosage. *Ann. Biol. clin.* 1967, **25**, 1211–1217.

Wiegand, R. G. & P. G. Sanders: Calculation of kinetic constants from blood levels of drugs. *J. Pharmacol. Exp. Therap.* 1964, **145**, 271–275.

Acta pharmacol. et toxicol. 1971, 29, 164–176.

From the Department of Medical Pharmacology, Uppsala University,
Biomedical Center, Uppsala, Sweden

Effect of Adrenalectomy, Corticosteroids and some other Anti-Inflammatory Agents, Salazopyrin®, Thyroxine and Vitamin A on the Exchangeable Sulphate Pool and on Sulphate Incorporation *in Vivo* into Costal Cartilage of the Mouse

By

Georg Herbai

(Received November 12, 1970)

Abstract: Drug effects on the size of the exchangeable sulphate pool and the incorporation rate into costal cartilage *in vivo* were studied by a new double isotope method in growing mice of the N. M. R. I. strain. Adrenalectomy caused a significant decrease both in the size of the sulphate pool and the rate of sulphation in cartilage. Hydrocortisone (a water soluble form), acetylsalicylic acid, oxyphenylbutazone and dimethyl-sulphoxide given in rather high doses had no effect on the sulphate pools or the sulphate incorporation rates. Hydrocortisone-acetate (in oil or suspension), prednisolone-acetate and dexamethasone all elicited a dose-dependent retardation of body growth and a considerable increase in the size both of the total sulphate pool and the pool per g body weight. The sulphation rate of the cartilage samples even at high doses was only moderately depressed by the steroids studied. Only prednisolone elicited a marked inhibition with a dose above 40 µg. Salazopyrin® (4 mg/day × 6) considerably decreased sulphate incorporation without affecting the size of the sulphate pool. Vitamin A caused a marked and dose-dependent increase in the sulphate pool and a moderate and dose-independent decrease in the sulphation of cartilage. *l*-Thyroxine up to a dose of 1 µg/day depressed sulphate incorporation, but a 10 times higher dose stimulated it. The sulphate pools displayed a graded elevation after increasing doses of *l*-thyroxine.

Key-words: Corticosteroids – anti-inflammatory agents – salazopyrin® – *l*-thyroxine – vitamin A – costal cartilage – sulphate pool – sulphate incorporation.

The incorporation of ^{35}S-sulphate into different kinds of connective tissue has been frequently used for the clarification of pharmacological aspects of mucopolysaccharide synthesis. The inhibitory effect of anti-inflammatory agents on chondroitin sulphate synthesis *in vitro* has been demonstrated in several previous communications (LAYTON 1951; BOSTRÖM & MÅNSSON 1955;

WHITEHOUSE & BOSTRÖM 1961; WHITEHOUSE & BOSTRÖM 1962). Furthermore, a similar inhibition of sulphate uptake into cartilage and skin mucopolysaccharides has also been presented *in vivo* after the administration of different anti-inflammatory drugs (BOSTRÖM & ODEBLAD 1953; SCHILLER & DORFMAN 1957; BOSTRÖM *et al.* 1964; WHITEHOUSE & BOSTRÖM 1965).

When ^{35}S sulphate is used for incorporation studies *in vivo*, there are a number of various biological sources of error, which must be taken into consideration during the evaluation of the results (LORENZEN 1963). One important factor in this connection is that different pharmacological agents may alter the size of the endogenous exchangeable sulphate pool of the animal and thus inadvertently change the specific activity of the ^{35}S-labelled material, which reaches the tissues after injection *in vivo*. Since micro-methods for sulphate determination in small biological samples were previously not available, a double isotope method has been developed for the simultaneous determination of the sulphate pool *in vivo* and the incorporation rate of sulphate into the costal cartilage of small laboratory animals. This allows correction for possible drug-induced alterations of the sulphate pool (HERBAI 1970a). In the present study the influence of some hormones and other drugs all of which have previously been reported as having actions on chondroitin sulphate synthesis, was re-evaluated by the method mentioned above.

Material and Methods

Animals.

Male and female mice of the N.M.R.I. strain were used throughout the study. They were either purchased from Anticimex AB, Sweden, or they were selected from the colony of our institute, which originated from animals obtained from Anticimex AB. In the drug dose-response experiments 4 weeks old animals were used with an average body weight of 16–19 g. All the animals were housed in plastic cages covered with wood shavings (5–7 mice/cage) and kept in a temperature controlled room at 25° and 12 · 12 day/night rhythm.

Diet.

The food consisted of commercial mouse pellets (Anticimex No. 213) *ad libitum* and free access to tap water. Vitamin supplement was given twice weekly (protovit®, Hoffmann-La Roche, Basel). Adrenalectomized mice were also supplied with fresh milk and a drinking solution containing 5 % glucose and 1 % NaCl.

Adrenalectomy was performed by the paravertebral approach under ether anaesthesia and the animals were kept 1 week before use. Controls were not sham-operated.

Isotopes.

Carrier-free ^{35}S-sulphate and ^{3}H-labelled phenol (specific activity 250 mci/mmol) were obtained from the Radiochemical Centre, Amersham, England. ^{3}H-phenol was dissolved in water and the stock solution was stored in the frozen state to minimize radiolysis. Its chemical integrity was repeatedly checked by thin-layer chromatography (TLC) and decomposition was never observed.

Drugs and hormones.

The following hormones were generously donated by Organon Co., Oss, Holland, and supplied by Pharmacia AB, Uppsala, Sweden: Hydrocortisone acetate, dexamethasone (fluormethylprednisolonum NFN) (both dissolved in olive oil). Hydrocortisone acetate was also supplied as a suspension (hydrocortal®; vehicle: benzyl alcohol, sodium carboxymethylcellulose, sorbimacrogole oleate), further diluted with 0.9 % NaCl. Prednisolone acetate was also used as a suspension (precortalon®; vehicle and dilution as for hydrocortal). Actocortin® (water soluble hydrocortisone-(21)-sodium phosphate-complex) was a gift of Cortec AB, Malmö, Sweden, and was diluted with distilled water. Vitamin A was obtained from Hoffmann-La Roche AG, Basel (arovit®: axerophthyl palmitate resp. 300,000 i.u. vitamin A). It was dissolved in olive oil. Nyegaard & Co. AS (Oslo, Norway) kindly supplied *l*-thyroxine sodium which was diluted with 0.9 % NaCl. Acetylsalicylic acid was purchased from ACO, Stockholm, and dimethylsulphoxide (Grade I) from Kemila AB, Stockholm. Oxyphenylbutazone (tanderil®) was a gift from Geigy AG, Basel, and was dissolved in 0.01 N-NaOH. Salazopyrin® (salicyl-azo-sulphapyridine, in the form of N-methyl-D-glucamine salt) was donated by Pharmacia AB, Uppsala, Sweden.

Measurement of the inorganic sulphate pool of the mouse and the incorporation rate of sulphate into costal cartilage.

The method has been described and evaluated elsewhere (HERBAI 1970a) but the principle of the technique and a short description of the standard procedure is given below. It is based on the formation of phenyl sulphate from injected phenol and its excretion in the urine. If ^3H-phenol and ^{35}S-sulphate with known specific activities are simultaneously injected intravenously into a mouse, a fraction of the phenol is sulphated and rapidly excreted in the urine. It can then be separated in the urine by TLC. The specific activity of the sulphate recovered as phenyl sulphate in the TLC-spot is different from that of the injected mixture because of the incorporation of sulphate from the endogenous inorganic sulphate pool. The size of the sulphate pool can be calculated from the $^3H/^{35}S$ ratio of the urinary phenyl sulphate and the specific activities of the injected compounds. After the urine sample for pool determination had been obtained, further incorporation of circulation ^{35}S-sulphate into cartilage was interrupted by a "quenching" injection of a large amount of unlabelled sodium sulphate. After the mouse had been killed a standardized amount of costal cartilage together with the osteochondral junctions was dissected and its content of ^{35}S measured. The amount of sulphate which had been incorporated into the sample during the experimental period was calculated from the size of the sulphate pool and the amount of ^{35}S incorporated into the cartilage specimen.

The standard procedure.

For the pharmacological evaluation of the drug effects, all compounds were administered in one daily dose intraperitoneally or subcutaneously for six days. The body weights of the mice were determined at the start of the injections and also before killing. Twenty-four hrs after the last drug dose the mice received an intravenous injection of 0.1 ml 0.9 % NaCl containing 20 µci ^{35}S with 96 µg (1 µmol) SO_4 and 20 µci ^3H-phenol with 376 µg (4 µmol) phenol. During the preparation of the injection mixture its exact ^3H and ^{35}S activity was determined by means of standardized reference solutions. Thirty minutes after the injection of the labelled material a urine sample (a few drops) was taken and the animals given 1 ml of 80 mg/ml $Na_2SO_4 \cdot 10 H_2O$ intraperitoneally. The urine sample was drawn into a glass tube and evaporated to

dryness in a vacuum desiccator. The dry residue of the urine was then mixed with 30 µl methanol containing 50 µg carrier phenyl sulphate/10 µl. A 10 µl sample was now transferred to the starting point of silica TLC-plates (DC-Fertigplatten, Kieselgel F_{254}, Merck A. G., Germany). The plates were developed by the ascending technique over a distance of 10 cm in a pre-equilibrated tank, using the following solvent system: 2-propanol, chloroform, methanol, water, 1 : 10 : 5 : 2 (v/v). The phenyl sulphate spot (R_F = 0.48) was then scraped directly into scintillation vials and the $^3H/^{35}S$ content measured in a Packard 3324 liquid scintillation spectrometer, after adding 5 ml of a scintillator solution of the dioxan type (HERBAI 1970b) to the vials. The costal cartilage sample from each mouse consisted of the caudal five pairs of attached ribs (No. III-VII) dissected free and cleaned from adherent tissue. Five osteochondral junctions were also included together with a small piece of the bony rib. After hot digestion in an oxidation mixture the ^{35}S content of the samples was determined as previously described (HERBAI 1970b).

Calculations.

The sulphate pools and the incorporation rates of sulphate into the cartilage samples were calculated by a computer program. Significance of differences between groups of data was tested by Student's t-test. In the case of body weight changes and sulphate pool values per g body weight, the statistics were calculated from data for the individual animals.

Results

Effect of adrenalectomy.

Six days after bilateral adrenalectomy a sulphate incorporation experiment was performed in operated and control mice. The results are presented in table 1. The adrenalectomized animals exhibited growth inhibition, a significant decrease in the sulphate pool and a pronounced inhibition of sulphate

Table 1.

Effect of adrenalectomy on the inorganic sulphate pool and rate of sulphate incorporation into costal cartilage of growing male mice. Figures are mean values ± S. E. M. R = rate of sulphate incorporation per costal cartilage sample. Figures in brackets are values in % of controls.

	n	Body weight g			Sulphate pool µg		R ng
		Before the operation	At death	Change	Total	Per g body weight	
Controls	8	18.2 ± 0.2	21.7 ± 0.4	3.5 ± 0.3	519 ± 31	24.0 ± 1.5	257 ± 11
Adrenal-ectomized	6	17.0 ± 0.3	17.4 ± 0.7	0 4 ± 0.7	256 ± 13 (42)	14.9 ± 1.1 (62)	136 ± 12 (53)

uptake into the costal cartilage samples (53 % of controls). It is obvious that a ^{35}S-sulphate incorporation experiment with tracer amounts of carrier SO_4 and without correction for the size of the endogenous sulphate pool would have missed the diminished incorporation rate of the adrenalectomized animals. The specific activity would have been erroneously high in the adrenalectomized mice and hence the incorporation rate would have been overestimated.

Effect of corticosteroids.

The results of treatment for 6 days with various corticosteroids are summarized in table 2. The experiments with any single substance were always done at the same time but the different substances were studied at long intervals. This explains the marked differences between the controls. Different doses of hydrocortisone (in the form of 3 different preparations), prednisolone and dexamethasone were studied. A moderate increase in body weight gain was seen after low doses of all the preparations except prednisolone, while a marked growth inhibition was elicited by the steroids in higher doses. One exception was the water-soluble and short-acting acid ester of hydrocortisone (actocortin®) which failed to affect any of the aspects investigated, possibly because of its rapid elimination. The low dose-induced slight elevation of body weights might be due to electrolyte and water retention. The sizes of the total sulphate pools of the mice were also significantly elevated by the steroids, the highest values being found after doses which yielded a weight gain stimulation.

The steroids also increased the size of the sulphate pool per g body weight. In this case the most marked differences were often found after the highest doses. The dissociation between effects on body weight, on sulphate pool per animal and on sulphate pool per g indicates that the causes of the sulphate pool alterations may be different at different dose levels.

As seen in table 2, the sulphate incorporation rates of cartilage, calculated after correction for the different specific activities due to pool alterations, were surprisingly little affected by the steroid treatment. There was a marked stimulation in one group with 8 μg hydrocortisone acetate in oil. This is a puzzling finding and needs to be repeated. It could be due to an erroneously high sulphate pool value. The only highly significant inhibition was elicited by prednisolone acetate in doses of 40 and 200 μg. This finding together with the sometimes marked changes in sulphate pool indicates that if the labelled material had been injected on a per animal or per g body weight basis, without information about the exchangeable sulphate pools of the animals, spuriously low sulphate incorporation values would have been found, merely due to a dilution effect and not to a really decreased sulphation rate of cartilage.

of growing mice.
A = Actocortin® (hydrocortisone) intraperitoneally, B = Hydrocortisone acetate (in oil) subcutaneously, C = Hydrocortal® (hydrocortisone acetate (in suspension) subcutaneously, D = Precortalon® (prednisolone acetate in suspension) subcutaneously, E = Dexamethasone (in oil) subcutaneously All drugs were given in one daily dose for 6 days. Controls received either oil (B, E) or 0.9 % NaCl (A, C, D). The figures indicate mean values ± S. E. M. n = number of animals per group.

Drug Sex	Dose µg	n	Body weight prior to treatment g	Weight change g	% of controls	Total sulphate pool µg	Change in % against controls	Sulphate pool per g b w. µg	Change in % against controls	Sulphate uptake per cartilage sample ng	% of controls
	Controls	4	17.5 ± 0.3	+ 4.1 ± 1.0	100	264 ± 5	0	12.3 ± 0.7	0	271 ± 10	100
A	125	4	16.5 ± 0.3	+ 4.8 ± 0.6	117	272 ± 13	+ 3	12.9 ± 1.1	+ 5	233 ± 35	86
	500	6	17.3 ± 0.2	+ 3.6 ± 0.4	88	284 ± 13	+ 8	13.6 ± 0.7	+ 11	227 ± 11	84*
♂	2000	4	16.6 ± 0.2	+ 3.1 ± 0.3	76	262 ± 9	− 0.8	13.4 ± 0.4	+ 9	256 ± 9	94
	Controls	5	18.6 ± 0.1	+ 6.5 ± 0.1	100	425 ± 20	0	16.9 ± 0.9	0	196 ± 13	100
	8	5	18.3 ± 0.1	+ 7.3 ± 0.2	112**	614 ± 26	+ 44***	24.0 ± 1.1	+ 42***	312 ± 13	159***
B	40	5	18.4 ± 0.1	+ 5.4 ± 0.7	83	608 ± 37	+ 43***	25.6 ± 1.8	+ 51***	205 ± 12	105
	200	5	18.6 ± 0.2	+ 6.0 ± 0.1	92*	541 ± 10	+ 27***	22.0 ± 0.4	+ 30***	214 ± 9	109
♂	1000	6	18.7 ± 0.2	+ 2.8 ± 0.4	43***	578 ± 23	+ 36***	27.0 ± 1.1	+ 60***	200 ± 12	102
	Controls	6	18.3 ± 0.3	+ 3.1 ± 0.3	100	360 ± 22	0	16.9 ± 1.2	0	191 ± 12	100
	8	6	18.7 ± 0.5	+ 2.9 ± 0.4	93	480 ± 24	+ 33**	22.3 ± 1.0	+ 32**	215 ± 11	113
C	40	6	19.0 ± 0.3	+ 3.6 ± 0.6	116	391 ± 13	+ 9	17.4 ± 0.7	+ 3	147 ± 10	77*
	200	6	19.4 ± 0.1	+ 2.1 ± 0.5	68	388 ± 22	+ 8	18.0 ± 0.9	+ 6.5	152 ± 15	80*
♀	1000	6	19.0 ± 0.3	− 0.8 ± 0.5	− 26***	391 ± 13	+ 9	21.5 ± 0.6	+ 27**	158 ± 4	83*
	Controls	6	17.5 ± 0.2	+ 3.4 ± 0.5	97	377 ± 21	0	18.0 ± 1.0	0	169 ± 10	100
	1.6	6	17.7 ± 0.4	+ 3.3 ± 0.4	76	451 ± 20	+ 20*	21.5 ± 0.6	+ 19**	160 ± 10	94
D	8	6	17.9 ± 0.5	+ 2.6 ± 0.3	15**	434 ± 16	+ 15*	21.1 ± 0.6	+ 17*	186 ± 8	110
	40	5	18.2 ± 0.4	+ 0.5 ± 0.5	100	391 ± 10	+ 4	20.9 ± 0.4	+ 16*	98 ± 7	58***
♀	200	7	18.1 ± 0.3	− 1.3 ± 0.6	− 38***	424 ± 26	+ 12	25.3 ± 1.4	+ 41***	110 ± 8	65***
	Controls	6	18.3 ± 0.3	+ 2.3 ± 0.3	100	221 ± 10	0	10.7 ± 0.4	0	193 ± 15	100
	0.1	6	18.2 ± 0.3	+ 3.4 ± 0.5	148*	318 ± 15	+ 44***	14.7 ± 0.6	+ 37***	196 ± 10	101
E	1	6	18.0 ± 0.3	+ 3.0 ± 0.2	130*	278 ± 9	+ 26***	13.2 ± 0.5	+ 23**	242 ± 9	125*
	10	6	18.2 ± 0.3	+ 0.9 ± 0.6	39*	269 ± 10	+ 22**	14.1 ± 0.3	+ 32***	150 ± 4	78*
♀	100	6	17.7 ± 0.4	− 0.1 ± 0.2	− 4***	264 ± 7	+ 19**	15.0 ± 0.4	+ 40***	170 ± 13	88

uptake into the costal cartilage samples (53 % of controls). It is obvious that a ^{35}S-sulphate incorporation experiment with tracer amounts of carrier SO_4 and without correction for the size of the endogenous sulphate pool would have missed the diminished incorporation rate of the adrenalectomized animals. The specific activity would have been erroneously high in the adrenalectomized mice and hence the incorporation rate would have been overestimated.

Effect of corticosteroids.

The results of treatment for 6 days with various corticosteroids are summarized in table 2. The experiments with any single substance were always done at the same time but the different substances were studied at long intervals. This explains the marked differences between the controls. Different doses of hydrocortisone (in the form of 3 different preparations), prednisolone and dexamethasone were studied. A moderate increase in body weight gain was seen after low doses of all the preparations except prednisolone, while a marked growth inhibition was elicited by the steroids in higher doses. One exception was the water-soluble and short-acting acid ester of hydrocortisone (actocortin®) which failed to affect any of the aspects investigated, possibly because of its rapid elimination. The low dose-induced slight elevation of body weights might be due to electrolyte and water retention. The sizes of the total sulphate pools of the mice were also significantly elevated by the steroids, the highest values being found after doses which yielded a weight gain stimulation.

The steroids also increased the size of the sulphate pool per g body weight. In this case the most marked differences were often found after the highest doses. The dissociation between effects on body weight, on sulphate pool per animal and on sulphate pool per g indicates that the causes of the sulphate pool alterations may be different at different dose levels.

As seen in table 2, the sulphate incorporation rates of cartilage, calculated after correction for the different specific activities due to pool alterations, were surprisingly little affected by the steroid treatment. There was a marked stimulation in one group with 8 μg hydrocortisone acetate in oil. This is a puzzling finding and needs to be repeated. It could be due to an erroneously high sulphate pool value. The only highly significant inhibition was elicited by prednisolone acetate in doses of 40 and 200 μg. This finding together with the sometimes marked changes in sulphate pool indicates that if the labelled material had been injected on a per animal or per g body weight basis, without information about the exchangeable sulphate pools of the animals, spuriously low sulphate incorporation values would have been found, merely due to a dilution effect and not to a really decreased sulphation rate of cartilage.

Table 3.

Effect of a *six* days treatment with salicyl-azo-sulphapyridine (salazopyrin®) on body weight, size of sulphate pool and incorporation rate of sulphate into costal cartilage of growing female mice. Controls received 0.9 % NaCl. The figures indicate mean values ± S. E. M.

	n	Body weight g			Sulphate pool μg		Sulphate uptake per cartilage sample ng
		Prior to treatment	At death	Change	Total	Per g body weight	
Controls	6	15.4 ± 0.5	18.2 ± 0.5	2 8 ± 0 3	459 ± 36	25.4 ± 2.4	181 ± 17***
Salazopyrin® 4 mg/mouse per day	7	15.9 ± 0.3	18.0 ± 0.5	2.2 ± 0 6	411 ± 19	23.0 ± 1.5	119 ± 4***

*** = $P < 0.001$.

Table 4.

Influence of vitamin A on body weight, sulphate pool and incorporation rate of sulphate into costal cartilage *of growing female mice.* Vitamin A *was given subcutaneously (in 0.1 ml olive oil) in one daily dose for 6 days. Controls received 0.1 ml olive* oil per day. The figures are mean values ± S. E. M. Each *group contained* 6 animals.

Daily dose i. u.	Body weight prior to treatment g	Weight change g	% of controls	Total sulphate pool µg	Change in % against controls	Sulphate pool per g b.w. µg	Change in % against controls	Sulphate uptake per cartilage sample ng	% of controls
Controls	17.2 ± 0.3	3.4 ± 0.5	100	180 ± 13	0	8.8 ± 0 6	0	193 ± 6	100
3750	17.6 ± 0 3	4.3 ± 0 6	126	211 ± 18	+ 17	9.6 ± 0 6	+ 9.1	150 ± 2.4	77***
7500	17.3 ± 0 2	1.8 ± 0.8	53	166 ± 16	− 8	8.9 ± 1.2	+ 1.1	135 ± 5	70***
15,000	17.4 ± 0.2	2.5 ± 0.3	73	245 ± 13	+ 36**	12.4 ± 0.6	+ 41***	145 ± 10	75**
30,000	17.6 ± 0 2	− 0.7 ± 0.8	− 21***	304 ± 26	+ 69***	18.0 ± 1.3	+ 104***	128 ± 11	66***

** = $P < 0.01$.
*** = $P < 0.001$.

Table 5.

Effect of a 6 days treatment with *l*-thyroxine on the *body weight, sulphate pool* and *incorporation* rate of sulphate into costal cartilage of growing female mice. The drug was diluted with physiological saline and given intraperitoneally in one daily dose (0.1 ml) for 6 days. Controls received 0.1 ml 0.9 % NaCl. The figures are mean values ± S. E. M. Each group contained 6 mice.

Daily dose µg	Body weight prior to treatment g	Weight change g	% of controls	Total sulphate pool µg	Change in % against controls	Sulphate pool per g b. w. µg	Change in % against controls	Sulphate uptake per cartilage sample ng	controls % of
Controls	22.2 ± 0.5	2.3 ± 0.4	100	366 ± 14	0	14.9 ± 0.6	0	244 ± 10	100
0.01	22.0 ± 0.7	2.4 ± 0.4	104	469 ± 18	+ 28***	19.2 ± 0.5	+ 29***	159 ± 12	65***
0.1	24.7 ± 0.9	2.9 ± 1.4	126	459 ± 43	+ 25*	16.9 ± 0.9	+ 13*	156 ± 11	64***
1.0	21.8 ± 0.9	2.1 ± 0.6	91	462 ± 23	+ 26**	19.4 ± 0.9	+ 30**	164 ± 11	67***
10	22.0 ± 0.5	2.1 ± 0.4	91	628 ± 24	+ 72***	26.2 ± 1.4	+ 76***	299 ± 13	123**

* = $P < 0.05$.
** = $P < 0.01$.
*** = $P < 0.001$.

Table 4.

Influence of vitamin A on body weight, sulphate pool and incorporation rate of sulphate into costal cartilage of growing female mice. Vitamin A was given subcutaneously (in 0.1 ml olive oil) in one daily dose for 6 days. Controls received 0.1 ml olive oil per day. The figures are mean values ± S. E. M. Each group contained 6 animals.

Daily dose i. u.	Body weight prior to treatment g	Weight change g	% of controls	Total sulphate pool μg	Change in % against controls	Sulphate pool per g b. w. μg	Change in % against controls	Sulphate uptake per cartilage sample ng	% of controls
Controls	17.2 ± 0.3	3.4 ± 0.5	100	180 ± 13	0	8.8 ± 0.6	0	193 ± 6	100
3750	17.6 ± 0.3	4.3 ± 0.6	126	211 ± 18	+ 17	9 6 ± 0 6	+ 9.1	150 ± 2.4	77***
7500	17.3 ± 0 2	1.8 ± 0.8	53	166 ± 16	− 8	8.9 ± 1.2	+ 1.1	135 ± 5	70***
15,000	17.4 ± 0.2	2.5 ± 0.3	73	245 ± 13	+ 36**	12.4 ± 0.6	+ 41***	145 ± 10	75**
30,000	17.6 ± 0.2	− 0.7 ± 0.8	− 21***	304 ± 26	+ 69***	18.0 ± 1.3	+ 104***	128 ± 11	66***

** = $P < 0.01$.
*** = $P < 0.001$.

incorporation observed in this study is mainly due to 5-ASA liberated after the injection of salazopyrin.

Previous studies on the effects of vitamin A on sulphate incorporation into connective tissue yielded conflicting results. In large doses, it was suggested, that vitamin A caused a splitting of the chondroitin sulphate chains from the protein core (THOMAS *et al.* 1960). An inhibition of chondrocyte activity *in vitro* has also been shown (MCELLIGOTT 1962). In rats vitamin A treatment caused a depression of ^{35}S incorporation in all tissues except the liver but the uronic acid content was only reduced in cartilage (MUKHERJI & BACHHAWAT 1966). In contrast, PERUMAL *et al.* (1966) found an increase of ^{35}S uptake into mucopolysaccharides of the intestine and colon. No correction for sulphate pool alterations were made in the studies quoted above, but the present results which showed a diminished chondroitin sulphate synthesis in cartilage after vitamin A treatment, confirmed the conclusions of MCELLIGOTT (1962) and those of MUKHERJI & BACHHAWAT (1966).

Thyroxine in a very large dose (200 μg per rat/day) was shown to decrease ^{35}S-sulphate incorporation in knee joint cartilage (DZIEWIATKOWSKI 1951). In contrast, LORENZEN (1961) obtained an increase of ^{35}S uptake into the mucopolysaccharides of the rabbit aorta after a dose of 50 μg/kg. In neither study was the sulphate pool checked. The dose-dependent biphasic effect reported here is difficult to explain but clearly shows how necessary it is to study a dose range and to correct for the size of the sulphate pool.

Acknowledgements

I am very grateful to Professor E. Bárány for his stimulating criticism and to Mrs. B. Jansson for excellent technical assistance.

This work was supported by grants from the Medical Faculty of Uppsala University and from the Nordisk Insulinfond, Gentofte, Denmark.

REFERENCES

Boström, H. & E. Odeblad: The influence of cortisone upon the sulphate exchange of chondroitin sulphuric acid. *Arkiv för kemi* 1953, 6, 39–42.

Boström, H. & B. Månsson: The action of salicylates and related compounds on the sulphate exchange of chondroitin sulphuric acid. *J. Pharmacol.* (Lond.) 1955, 7, 185–190.

Boström, H., K. Berntsen & M. W. Whitehouse: Biochemical properties of anti-inflammatory drugs – II. Some effects on sulphate-^{35}S metabolism *in vivo*. *Biochem. Pharmacol.* 1964, 13, 413–420.

Dziewiatkowski, D. D.: Effect of thyroxine and thiouracil on S^{35} deposition in articular cartilage. *J. biol. Chem* 1951, 189, 717–727.

Hanngren, A., E. Hansson, N. Swartz & S. Ullberg: Distribution and metabolism of salicyl-azo-sulfapyridine – I. A study with C^{14}-salicyl-azo-sulfapyridine and C^{14}-5 amino-salicylic acid. *Acta med. scand.* 1963a, 173, 61–72.

Hanngren, A., E. Hansson, N. Swartz & S. Ullberg: Distribution and metabolism of salicyl-azo-sulfapyridine -II. A study with S35-salicyl-azo-sulfapyridine and S35-sulfapyridine. *Acta med. scand.* 1963b, 173, 391–399.

Helander, S.: On the concentrations of some sulfanilamide derivates in different organs and tissue structures. Thesis. *Acta physiol. scand.* 1945, 10, Suppl. 29, 1–103.

Helander, S.: *On the distribution of some salicylic acid derivatives in the tissues. Acta pharmacol. et toxicol.* 1950, 6, 97–106.

Herbai, G.: *A double isotope method for determination of the miscible inorganic sulphate pool of the mouse applied to in vivo studies of sulphate incorporation* into costal cartilage. *Acta physiol. scand.* 1970a, 80, 470–491.

Herbai, G.: Incorporation and disappearance of sulfate in different regions of mouse and rat costal cartilage *in vivo* and *in vitro. Acta physiol. scand.* 1970b, 79, 541–551.

Layton, L. L.: Effect of cortisone upon chondroitin sulfate synthesis by animal tissues. *Proc. Soc. Exp. Biol.* (N. Y.) 1951, 76, 596–598.

Lerner, L. J., A. Bianchi, A. R. Turkheimer, F. M. Singer & A. Borman: Anti-inflammatory steroids: potency, duration and modification of activities. *Ann. N. Y. Acad. Sci.* 1964, 116, 1071–1077.

Lorenzen, I.: *Vascular connective tissue under the influence of thyroxine II. Effect of* l-thyroxine on the aortic wall in rabbits. *Acta endocrin.* (Kbh.) 1961, 37, 183–190.

Lorenzen, I.: *Experimental arteriosclerosis.* Biochemical and morphological changes induced by adrenaline and thyroxine. Thesis. Munksgaard, Copenhagen 1963.

McElligott, T. F.: Decreased fixation of sulphate by chondrocytes in hypervitaminosis A. *J. Path. Bact.* 1962, 83, 347–355.

Mukherji, B. & B. K. Bachhawat: Role of vitamin A in sulphate metabolism: Part II – Studies on sulphate incorporation into mucopolysaccharides in hypervitaminotic A rats. *Indian J. Biochem* 1966, 3, 1–4.

Perumal, A. S., T. S. A. Samy, M. R. Lakshmanan, F. B. Jungalwala & P. B. R. Rao: *Biosynthesis of sulfated mucopolysaccharides in relation to vitamins A and K. Biochim. biophys. Acta* 1966, 124, 95–100.

Sarett, L. H., A. A. Patchett & S. L. Steelman: The effects of structural alteration on the anti-inflammatory properties of hydrocortisone. In: *Progress in drug research, Vol. 5,* 1963, 11–153, Ed: E. Jucker. Birkhäuser Verlag, Basel und Stuttgart.

Schiller, S. & A. Dorfman: The metabolism of mucopolysaccharides in animals: The effect of cortisone and hydrocortisone on rat skin. *Endocrinology,* 1957, 60, 376–381.

Thomas, L., R. T. McCluskey, J. L. Potter & G. Weissmann: Comparison of the effect of papain and vitamin A on cartilage. I. The effects in rabbits. *J. exp. Med.* 1960, **111**, 705–718.

Whitehouse, M. W. & H. Boström: *Studies on the action of some anti-inflammatory agents in inhibiting the biosynthesis of mucopolysaccharide sulphates. Biochem. Pharmacol.* 1961, 7, 135–150.

Whitehouse, M. W. & H. Boström: The effect of some anti-inflammatory (anti-rheumatic) drugs on the metabolism of connective tissues. *Biochem. Pharmacol.* 1962, 11, 1175–1201.

Whitehouse, M. W. & H. Boström: Biochemical properties of *anti-inflammatory drugs* – VI. The effects of chloroquine (resochin), mepacrine *(quinacrine) and some* of their potential metabolites on cartilage *metabolism and* oxidative phosphorylation. *Biochem. Pharmacol.* 1965, 14, 1173–1184.

Acta pharmacol. et toxicol. 1971, 29, 177–193.

From the Department of Medical Pharmacology, Uppsala University, Biomedical Center, Uppsala, Sweden

Effect of Pregnancy, Castration, Testosterone, Ethisterone, Oestradiol Benzoate and Stilboestrol on the Exchangeable Sulphate Pool and on Sulphate Incorporation *in Vivo* into Costal Cartilage of the Mouse

By

Georg Herbai

(Received November 26, 1970)

Abstract: The influence of different endocrine conditions and the effects of sex hormones on the sulphate incorporation rate *in vivo* into costal cartilage were studied in mice by a method which allows a correction of the sulphation rates for alterations in the exchangeable sulphate pools of the animals Oestrous cycle variations had no measurable effect, but castration of both male and female mice decreased the size of the sulphate pool and sulphation rate of cartilage, while pregnancy had the opposite effect. Testosterone propionate (dose range: 1 µg–1 mg/day × 6) and ethisterone (dose range: 40 µg–5 mg/day × 6) stimulated weight gain but had a rather weak effect on the sulphate pools and incorporation rates. Oestradiol benzoate and diethylstilboestrol given to growing mice in doses of 1 µg–1 mg/day × 6 caused a dose-dependent growth retardation and a decrease in the size of the total sulphate pool and that per g body weight. The drugs also elicited a graded inhibition of sulphation rate of cartilage in both sexes. Stilboestrol (20 µg/day × 6) also inhibited growth and decreased sulphation rate in 12-days old suckling mice. Adult male and female animals treated with oestradiol benzoate in the doses described above, displayed only small weight changes but a marked and dose-dependent decrease in the sulphation rate. Inhibition of sulphate incorporation rate always went together with growth retardation in growing mice but the former effect was produced by lower doses of oestradiol benzoate.

Key-words. Sex hormones – sulphate incorporation – costal cartilage – exchangeable sulphate pool – body growth rate.

The influence of gonadal hormones on growth and skeletal development has been previously studied by many investigators. It is well established that oestrogens cause retardation of body growth in many different species (for an extensive review see SILBERBERG & SILBERBERG 1956). Growth rate was also shown to be affected by testosterone (SWANSON & VAN DER VERFF 1963)

and by progesterone (HERVEY & HERVEY 1965). Since sulphation of mucopolysaccharides is an important reaction during the growth process, several investigators had previously studied the influence of sex hormones on ^{35}S-sulphate uptake into different kinds of connective tissue *in vivo* (DZIEWIATKOWSKI *et al.* 1957; PRIEST *et al.* 1960; SALMON *et al.* 1963; NAKAMURA & MASUDA 1966; BERNTSEN 1968). However, it has been emphasized and reviewed in a previous communication (LORENZEN 1963) that incorporation experiments with ^{35}S *in vivo* are associated with a number of biological sources of error which may influence the correctness of the results. In the studies on sex hormones and sulphate incorporation quoted above, the specific activity of the circulating ^{35}S was not controlled, since micro-methods for sulphate determination in small biological samples were not available. This lack of information might have been a source of error in the interpretation of the results, since recent investigations have shown that treatment with different hormones can markedly influence the size of the exchangeable sulphate pool of the animals (HERBAI 1971a & b). The purpose of the present study was to evaluate the influence of some endocrine conditions and measure the effect of sex hormones on sulphate incorporation *in vivo* into cartilage, after correction for the variations in the sulphate pool of the individual animals.

Material and methods

Animals.

Male and female mice of the N. M. R. I. strain were used throughout the study. They were either purchased from Anticimex AB, Sweden, or selected from the colony of the Department of Pharmacology, which originated from Anticimex AB. In most experiments rapidly growing 4 weeks old animals were used but in some experiments 12-days old suckling mice or in others, slowly growing adult mice were studied. In one experiment pregnant mice in the second part of pregnancy were used. The stage of pregnancy was checked at autopsy by the foetus weights, and only those pregnant mice which had foetuses weighing above 0.5 g were included. All the animals were housed in plastic cages covered with wood shavings (5–8 mice/cage) and kept in a room with a constant temperature of 25° and 12 : 12 day/night rhythm. They were supplied with commercial mouse pellets (Anticimex no. 213) *ad lib.* and had free access to tap water. Vitamin supplement was given twice weekly (protovit®, Hoffman-La Roche, Basel). Ovariectomy and orchidectomy were performed in immature mice (2 weeks old) and the animals were kept for 2 months before use. The controls were not sham-operated. The stage of the oestrous cycle was checked by vaginal smears.

Isotopes.

Carrier-free ^{35}S-sulphate and ^{3}H labelled phenol (specific activity: 250 mci/mmol) were obtained from the Radiochemical Centre, Amersham, England. ^{3}H-phenol was dissolved in water and a stock solution was stored in the frozen state. Its chemical purity was checked by thin-layer chromatography (TLC) and radiolysis was never observed.

Hormones.

Testosterone propionate and oestradiol benzoate were donated by Organon Co., Oss, Holland, and supplied by Pharmacia AB, Uppsala, Sweden. They were dissolved in and further diluted with olive oil. Ethisterone and oestradiol-17 β (free alcohol) were purchased from Sigma Chem. Co., St. Louis, Mo., U.S.A. Ethisterone was dissolved in a 10 % propylene glycol-water solution and oestradiol in olive oil. Diethylstilboestrol was obtained from ACO, Stockholm, Sweden, and dissolved in olive oil. The animals in the control groups received only olive oil and in the ethisterone experiment, 10 % propylene glycol-water mixture.

Measurement of the exchangeable sulphate pool of the mouse and incorporation rate of sulphate into costal cartilage.

The details and evaluation of the method have been described elsewhere (HERBAI 1970a) but a short presentation of the principle and the course of the routine procedure is given in a previous communication in this journal (HERBAI 1971b).

Results

Influence of the oestrous cycle, pregnancy and castration.

Before making a closer study of the effects of injected sex hormones, the influence on the size of sulphate pool and incorporation rate of some basic endocrine conditions which are related to sex hormones was investigated. The results are summarized in table 1. Thirty-three sexually mature female mice were selected with regard to their actual oestrous stage and three groups of animals, in oestrous, pro-oestrous and di-oestrous stage respectively, were subjected to a sulphate incorporation experiment. As can be seen from table 1, the cycle did not influence the size of sulphate pool or the incorporation rate of sulphate into costal cartilage.

The middle part of table 1 shows that pregnant mice showed highly significant increase in the size of the sulphate pool. Apart from the body weight dependent elevation of the total sulphate pool, the pool per g body weight was also significantly increased. Furthermore, a probably significant increase in the sulphate incorporation rate of cartilage was recorded in pregnant mice.

The long-term effects of castration in male and female mice are presented in the lowest part of table 1. The growth rate was nearly normal in ovariectomized female mice but was markedly decreased in castrated males. Both the total sulphate pool and that per g body weight of the mice were diminished after castration in both sexes. Likewise the sulphation rate of costal cartilage was significantly decreased in both male and female mice.

Effect of testosterone propionate and ethisterone.

Androgenic effects were studied using testosterone propionate and ethisterone, the latter being a potent gestagen with some androgenic action. As can

be seen from table 2 both compounds stimulated the growth rate of the animals. The size of the total sulphate pool and that per g body weight was only slightly influenced by these hormones and no clear dose-response relation was seen. Increasing doses of testosterone did not stimulate the rate of sulphate incorporation; on the contrary 1 mg per day significantly diminished this. Ethisterone in a dose of 40 μg (the lowest studied dose) caused a significant elevation of the sulphate incorporation rate, but after higher doses the stimulation was decreased.

Table 1.

Effect of oestrous cycle, pregnancy and castration on the inorganic sulphate pool and incorporation rate of sulphate into costal cartilage of sexually mature mice. Figures show mean values ± S. E. M. Time between castration and experiment 2 months. All pregnant mice contained foetuses of at least 0.5 g, as confirmed at autopsy, indicating that they were selected in a relatively late part of pregnancy. Significance test against controls: *) = $P < 0.05$, **) = $P < 0.002$, ***) = $P < 0.001$. The experiments A, B and C were run on different occasions and the figures are not immediately comparable.

	Hormonal conditions	n	Body weight at death g	Sulphate pool μg		Rate of sulphate incorporated per cartilage sample ng	% of control
				Total	Per g body weight		
A	Di-oestrous	8	28.1 ± 0.5	378 ± 32	13.4 ± 1.1	149 ± 6	100
A	Pro-oestrous	8	27.2 ± 0.9	385 ± 20	14.3 ± 1.0	152 ± 10	102
A	Oestrous	9	27.1 ± 0.4	363 ± 27	13.3 ± 0.9	141 ± 7	94.6
B	Controls	3	30.2 ± 1.6	281 ± 22	9.4 ± 1.2	76.8 ± 2.1	100
B	Pregnant mice	5	42.5 ± 4.4*	636 ± 33***	15.4 ± 1.2**	86.5 ± 3.2	112.5*
C	Normal females	7	32.3 ± 0.6	315 ± 34	9.7 ± 1.1	104.2 ± 4.6	100
C	Ovariectomized	7	31.7 ± 0.8	242 ± 18	7.6 ± 0.5*	76.8 ± 4.9	74**
C	Normal males	7	39.3 ± 0.6	445 ± 18	11.3 ± 0.4	99.0 ± 3.5	100
C	Orchidectomized	7	33.6 ± 0.9***	332 ± 27**	9.8 ± 0.6*	66.4 ± 4.2	67***

Table 2.

Effect of different doses of testosterone propionate and ethisterone on weight-gain, size of sulphate pool and incorporation of sulphate into costal cartilage of growing female mice. The drugs were given in one daily dose for 6 days. Figures show mean values ± S. E. M. Significance test against controls: *) = $P < 0.05$, **) = $P < 0.01$. The experiments with testosterone and ethisterone were run on different occasions and the figures are not immediately comparable.

Drug	Daily dose	n	Body weight g		Change % of controls	Sulphate pool µg		Sulphate incorporated per cartilage sample ng	% of controls
			Prior to treatment	Change		Total	Per g body weight		
Testosterone propionate s. c.	Controls	6	17.8 ± 0.3	2.5 ± 0.2	100	321 ± 15	15.7 ± 0.5	272 ± 19	100
	1 µg	6	17.8 ± 0.3	2.9 ± 0.4	116	378 ± 24*	18.4 ± 1.2*	306 ± 21	112
	10 µg	6	17.8 ± 0.4	3.5 ± 0.3	140**	358 ± 20	16.8 ± 0.9	268 ± 21	98
	100 µg	6	17.8 ± 0.4	4.1 ± 0.5	164**	358 ± 11*	16.4 ± 0.6	273 ± 14	100
	1 mg	7	17.2 ± 0.4	3.5 ± 0.4	140*	332 ± 12	16.1 ± 0.5	212 ± 7	78**
Ethisterone s. c.	Controls	6	17.2 ± 0.2	1.7 ± 0.4	100	156 ± 15	8.2 ± 0.7	154 ± 10	100
	40 µg	6	17.3 ± 0.3	2.8 ± 0.5	165	228 ± 26*	11.4 ± 1.4*	217 ± 17	141**
	200 µg	6	17.5 ± 0.4	2.1 ± 0.5	124	181 ± 9	9.3 ± 0.6	193 ± 14	125*
	1 mg	6	17.7 ± 0.3	2.0 ± 0.7	118	184 ± 11	9.4 ± 0.7	141 ± 5	91
	5 mg	7	17.4 ± 0.3	2.6 ± 0.4	153	215 ± 39	10.6 ± 1.9	176 ± 15	114

be seen from table 2 both compounds stimulated the growth rate of the animals. The size of the total sulphate pool and that per g body weight was only slightly influenced by these hormones and no clear dose-response relation was seen. Increasing doses of testosterone did not stimulate the rate of sulphate incorporation; on the contrary 1 mg per day significantly diminished this. Ethisterone in a dose of 40 μg (the lowest studied dose) caused a significant elevation of the sulphate incorporation rate, but after higher doses the stimulation was decreased.

Table 1.

Effect of oestrous cycle, pregnancy and castration on the inorganic sulphate pool and incorporation rate of sulphate into costal cartilage of sexually mature mice. Figures show mean values ± S. E. M. Time between castration and experiment 2 months. All pregnant mice contained foetuses of at least 0 5 g, as confirmed at autopsy, indicating that they were selected in a relatively late part of pregnancy. Significance test against controls: *) = P < 0 05, **) = P < 0.002, ***) = P < 0.001. The experiments A, B and C were run on different occasions and the figures are not immediately comparable.

	Hormonal conditions	n	Body weight at death g	*Sulphate pool μg*		Rate of sulphate incorporated per cartilage sample ng	*% of control*
				Total	*Per g body weight*		
	Di-oestrous	8	28.1 ± 0.5	378 ± 32	13.4 ± 1.1	149 ± 6	100
A	Pro-oestrous	8	27.2 ± 0 9	385 ± 20	14.3 ± 1.0	152 ± 10	102
	Oestrous	9	27.1 ± 0.4	363 ± 27	13.3 ± 0.9	141 ± 7	94 6
B	Controls	3	30.2 ± 1.6	281 ± 22	9.4 ± 1.2	76.8 ± 2.1	100
	Pregnant mice	5	42.5 ± 4.4*	636 ± 33***	15.4 ± 1.2**	86.5 ± 3.2	112.5*
C	Normal females	7	32.3 ± 0.6	315 ± 34	9.7 ± 1.1	104.2 ± 4.6	100
	Ovariectomized	7	31.7 ± 0.8	242 ± 18	7.6 ± 0.5*	76 8 ± 4.9	74**
	Normal males	7	39.3 ± 0.6	445 ± 18	11.3 ± 0.4	99.0 ± 3.5	100
	Orchidectomized	7	33.6 ± 0.9***	332 ± 27**	9.8 ± 0 6*	66.4 ± 4 2	67***

Table 3.

Effect of increasing doses of oestradiol benzoate on body growth rate, size of sulphate pool and incorporation rate of sulphate into costal cartilage of growing male and female mice. The homone was given in one daily dose for 6 days. Figures show mean values ± S. E. M. n = number of animals per group. Significance tests against controls: *) = $P < 0.05$, **) = $P < 0.01$, ***) = $P < 0.001$. The experiments on males and females were run on different occasions and the figures are not immediately comparable.

Sex	Daily dose µg	n	Body weight prior to treatment g	Weight change		Total sulphate pool µg	Change in % against controls	Sulphate pool per g b. w. µg	Change in % against controls	Incorporated sulphate per cartilage sample ng	% of controls
				g	% of controls						
Males	controls	8	16.3 ± 0.2	+ 5.5 ± 0.4	100	359 ± 22	0	16.5 ± 1.0	0	182 ± 11	100
	1	4	16.3 ± 0.4	+ 6 8 ± 0.2	124*	376 ± 22	+ 5	16.3 ± 1.1	−1.2	196 ± 19	108
	10	4	16.0 ± 0.4	+ 4.2 ± 0.2	76*	266 ± 17	−26*	13.1 ± 0.7	−21*	144 ± 4	79*
	100	4	16.0 ± 0.3	+ 1.9 ± 0.5	34***	219 ± 6	−39***	12.3 ± 0.7	−25*	82 ± 4	45***
	1000	4	16.0 ± 0.2	+ 1.6 ± 0.7	29***	220 ± 6	−39***	12.5 ± 0.4	−24*	85 ± 4	46***
Females	controls	4	16 6 ± 0.3	+ 3.1 ± 0.6	100	248 ± 16	0	12.6 ± 0.8	0	171 ± 15	100
	1	4	16.4 ± 0.2	+ 4 2 ± 0.4	135	276 ± 17	+11	13.3 ± 0.7	+ 6	144 ± 13	84
	10	3	17.5 ± 0.3	+ 2.3 ± 1.0	74	210 ± 44	−15	10.5 ± 1.9	−17	89 ± 5	52**
	100	4	17.4 ± 0.1	+ 0.7 ± 0.7	23*	89 ± 23	−64***	4.9 ± 1.3	−61***	52 ± 5	31***
	1000	4	16.8 ± 0.4	+ 0.9 ± 0.5	29*	103 ± 31	−58***	5.8 ± 1.8	−54**	58 ± 5	34***

Table 4.

Effect of increasing doses of oestradiol benzoate on body growth rate, size of sulphate pool and incorporation rate of sulphate into costal cartilage *of* adult male and female mice. The hormone was given in one daily dose for 6 days. Figures show mean values ± S. E. M. n = number of mice per group. Significance test against controls: *) = $P < 0.05$, **) = $P < 0.01$, ***) = $P < 0.001$. *The experiments* on males and females were run on different occasions and the *figures are not immediately* comparable.

Sex	Daily dose μg	n	Body weight prior to treatment g	Weight change		Total sulphate pool μg	Change in % against controls	Sulphate pool per g b. w. μg	Change in % against controls	Incorporated *sulphate per* cartilage sample ng	% of controls
				g	% of controls						
Males	controls	5	36.3 ± 0.9	1 0 ± 0.3	100	457 ± 50	0	12.3 ± 1.4	0	102 ± 4	100
	1	5	36.0 ± 1.0	2.5 ± 0.5	250*	460 ± 34	+0.7	12.0 ± 0.8	−2.4	102 ± 2	100
	10	5	35.0 ± 1.3	3.1 ± 0.3	310***	357 ± 32	−22	9.4 ± 1.0	−24	81 ± 2	79**
	100	5	34.3 ± 0 8	0.8 ± 0.7	80	276 ± 39	−40*	7.9 ± 1.2	−36*	65 ± 5	64***
	1000	5	35.9 ± 0.6	−1.9 ± 0.2	−190***	295 ± 46	−35*	8.7 ± 1.4	−29	52 ± 3	51***
Females	controls	5	26.5 ± 0.9	1.2 ± 0.5	100	243 ± 34	0	8.8 ± 1.2	0	104 ± 10	100
	1	4	26.6 ± 0.8	2.6 ± 0.8	217	270 ± 42	+11	9.1 ± 1.0	+3.4	74 ± 4	71*
	10	6	25.8 ± 0.6	3.0 ± 0.5	250*	297 ± 11	+22	10.3 ± 0.3	+17	35 ± 2.5	33***
	100	5	26.0 ± 0.5	0 8 ± 0.4	67	243 ± 25	0	9.2 ± 1.1	+4.5	40 ± 3	39***
	1000	4	27.6 ± 0.3	−0 4 ± 0.7	− 33*	162 ± 44	−33	5.9 ± 1.6	−33	27 ± 2	26***

Table 5.

Effect of increasing doses of diethylstilboestrol on body growth rate, size of sulphate pool and incorporation rate of sulphate into costal cartilage of growing male and female mice. The drug was given in one daily dose for 6 days. Figures show mean values ± S. E. M. n = number of mice per group. Significance test against controls: *) = $P < 0.05$, **) = $P < 0.01$, ***) = $P < 0.001$. The experiments on males and females were run on different occasions and the figures are not immediately comparable.

Sex	Daily dose µg	n	Body weight prior to treatment g	Weight change		Total sulphate pool µg	Change in % against controls	Sulphate pool per g b. w. µg	Change in % against controls	Incorporated sulphate per cartilage sample ng	% of controls
				g	% of controls						
Males	controls	6	15.7 ± 0.3	6.2 ± 0.8	100	342 ± 35	0	15.6 ± 1.5	0	416 ± 32	100
	1	6	15.9 ± 0.2	5.6 ± 0.6	90	329 ± 35	− 4	15.2 ± 1.5	− 3	373 ± 22	90
	10	5	15.5 ± 0.3	3.3 ± 0.6	53**	233 ± 16	−32*	12.5 ± 1.1	−20	189 ± 15	45***
	100	5	15.2 ± 0.2	1.0 ± 0.5	16***	230 ± 13	−33**	14.3 ± 1.2	− 8	137 ± 7	33***
	1000	6	15.6 ± 0.3	0.1 ± 0.4	1.6***	178 ± 13	−48***	11.3 ± 0.7	−28*	88 ± 9	21***
Females	controls	6	14.8 ± 0.2	6.0 ± 0.3	100	482 ± 15	0	23.1 ± 0.6	0	342 ± 19	100
	1	6	14.9 ± 0.1	6.0 ± 0.3	100	512 ± 28	+ 6	24.5 ± 1.5	+ 6	318 ± 7	93
	10	6	14.7 ± 0.1	4.3 ± 0.4	72**	471 ± 16	− 2	24.8 ± 0.9	+ 7	168 ± 15	49***
	100	6	14.8 ± 0.1	1.5 ± 0.5	25***	397 ± 12	−18**	24.4 ± 0.9	+ 6	134 ± 8	39***
	1000	7	14.7 ± 0.1	0.8 ± 0.3	13***	436 ± 17	− 9*	28.1 ± 0.8	+22***	102 ± 7	30***

basis were decreased, but in the female mice the sulphate pool per g value was practically unchanged after increasing doses of stilboestrol. An exception was the decreased pool found after 1 mg of stilboestrol. A comparison between stilboestrol and oestradiol benzoate (tables 5 & 3) is of doubtful value, since the two substances were tested at long intervals and the control values differ markedly between the groups.

Effect of stilboestrol in immature suckling mice.

It has been reported that in immature rats with body weights of less than 100 g, stilboestrol administration failed to inhibit the rate of body growth in contrast to its effect in rats above 100 g. (NOBLE 1938; NOBLE 1939). In order to test the sensitivity of immature mice, 12-days old suckling mice of unspecified sex were injected with 20 µg stilboestrol for 6 days, after which an incorporation experiment was performed using a reduced amount of the injection mixture. Table 6 shows the results. The stilboestrol treated mice exhibited complete arrest of growth, the final body weight in fact being lower than that before the hormone administration. The control group grew about 2 g during the same time. The total sulphate pools were somewhat lower and those per g body weight somewhat higher in the stilboestrol treated group. These results were, however, not statistically significant. The sulphate incorporation rate was profoundly decreased after stilboestrol treatment. In contrast to the findings of NOBLE in the rat (1938 & 1939) stilboestrol is effective even in very young mice.

Table 6.

[illegible] of stilboestrol treatment for 6 days on weight-gain, size of the inorganic sulphate pool and rate of sulphate incorporation into costal cartilage in 12-days old suckling mice of unspecified sex. The figures show mean values ± S. E. M. n = number of mice per group.

	n	Body weight g			Sulphate pool µg		Sulphate incorporated per cartilage sample ng
		Prior to treatment	At death	Change	Total	Per g body weight	
Controls	9	6.3	8.3 ± 0.2	+2.0 ± 0.2	43.0 ± 4.7	5.2 ± 0.6	160 ± 8
20 µg Stilboestrol per day	9	6.3	5.8 ± 0.1	−0.5 ± 0.1***	34.8 ± 2.6	6.0 ± 0.4	65 ± 4***

*** = $P < 0.001$.

Table 7.

Influence of the duration of oestrogen treatment on body weight gain, size of sulphate pool and rate of sulphate incorporation into costal cartilage of growing male mice. The figures are mean values ± S. E. M. Each group contained 4 mice. The second group in the table received 100 μg oestradiol-17 β (OE) for two days and all other groups were injected with 100 μg oestradiol benzoate (OB) daily for a varying number of days before the incorporation experiment. Significance test against the control group: *) = $P < 0.05$, **) = $P < 0.01$, ***) = $P < 0.001$.

Duration of treatment days	Body weight g			Sulphate pool μg		Sulphate incorporated per cartilage sample ng	% of controls
	Day 0	Day 8	Change	Total	Per g body weight		
Controls	17.8 ± 0.2	23.6 ± 0.6	5.8 ± 0.5	336 ± 36	14.3 ± 1.7	307 ± 23	100
OE 2	17.8 ± 0.6	22.7 ± 1.2	5.0 ± 0.6	196 ± 20**	8.6 ± 0.6**	178 ± 21	58**
OB 2	18.2 ± 0.2	24.4 ± 0.6	6.2 ± 0.6	306 ± 31	12.6 ± 1.4	279 ± 27	91
OB 3	18.3 ± 0.4	24.8 ± 0.6	6.4 ± 0.8	431 ± 28*	17.4 ± 1.1	186 ± 17	60**
OB 4	18.1 ± 0.2	22.4 ± 0.9	4.3 ± 0.9	378 ± 51	16.8 ± 2.0	155 ± 12	50***
OB 5	18.1 ± 0.4	21.2 ± 1.1	3.1 ± 0.9*	284 ± 29	13.3 ± 0.9	116 ± 9	38***
OB 6	18.7 ± 0.5	19.4 ± 0.9	0.7 ± 0.6***	264 ± 42	13.8 ± 2.6	96 ± 6	31***
OB 7	18.6 ± 0.5	18.5 ± 0.2	−0.1 ± 0.3***	237 ± 49	12.8 ± 2.5	83 ± 8	27***

Influence of the duration of oestrogen treatment.

Eight groups of growing male mice were selected. Six groups received a daily dose of 100 μg oestradiol benzoate for 2–7 days, the last dose being given 24 hrs before the experiment. Thus, the six groups were under the influence of oestradiol for 48, 72, and so on, hours, respectively before the experiment. One separate group received the same dose of free oestradiol-17β for two consecutive days. The control group received oil only, 7 times. The incorporation experiment was done simultaneously in all the groups, so that a common control group could be used. The results are summarized in table 7. The free alcohol form of oestradiol, which is probably more quickly absorbed and more short-acting, influenced the weight gain of the mice only slightly but significantly decreased the size of the total sulphate pool and also that per g body weight. It also caused a significant inhibition of sulphate incorporation into the cartilage samples. In a separate experiment (not shown in the table), a single dose of 100 μg free oestradiol given 24 hours before the experiment also caused an insignificant decrease to 78 %.

With oestradiol benzoate the effect appeared somewhat later since as can be seen from the third row in table 7, two injections of the hormone did not cause a significant fall in the incorporation rate of sulphate. The sulphate pool values were only slightly and irregularly affected in this experiment. The sulphate incorporation rate of cartilage exhibited a graded and progressive decrease after increasing length of treatment. Whether this is due to an accumulation of the hormone itself is not clear. At any rate the experiment shows that, at least for 7 days, no escape from the inhibition occurs.

Discussion

If the results presented in different tables are compared with each other it becomes evident that major or minor differences exist between the control groups with regard to weight gain, size of sulphate pool and rate of sulphate incorporation into cartilage. Furthermore, the hormone induced changes sometimes differed despite similar treatment. When 6 × 100 μg oestradiol benzoate was given to male growing mice in the experiments of table 3, a significant decrease in the sulphate pool values occurred, while in those of table 7 only a moderate and non-significant decrease was observed. These discrepancies between some control values and also the inconsistency of some drug-induced changes are puzzling. The following is an attempt at an explanation. The different experiments presented in this study were performed over the course of several years, but each separate study involving different doses of a drug and its control was run at the same time. The animals were all of the same strain obtained from the same colony. The food pellets were of the same make and – as far as is known – composition. The mice were

selected for each experiment using the body weight as guide. It is possible that an uncontrolled factor (see below) can have affected the growth rate and thus that animals with similar body weights were in fact of different ages in the different experiments. The differences in the incorporation rate observed between different controls could thus be due to variations in age, since, as has been shown previously, the incorporation rate of sulphate is rather age sensitive (HERBAI 1971a).

The main factor which could be responsible for all the variations quoted above could be associated with the wood shavings in the cages. All mice were kept on wood shavings, sometimes for a long time as during the breeding of the colony of the institute, and the shavings were obtained from different sources and of different quality. It has recently become clear that such wood shavings as a rule contain fungistatic agents such as pentachlorophenol. There is well documented evidence in the literature that halogenated cyclic hydrocarbons such as chlordane, DDT and aldrin, profoundly stimulate the synthesis of liver microsomal enzyme systems and, as has been shown, significantly alter many different effects of progesterone, testosterone and oestrogens (CONNEY *et al.* 1965; CONNEY *et al.* 1966; LEVIN *et al.* 1968). Furthermore, in an extensive reveiew of this subject it is concluded that comparisons between drug effects might be influenced by the content of liver microsomal enzyme inducers contained in different bedding chips in animal cages (CONNEY 1967). It is therefore quite possible that such a factor can be responsible for the discrepancies mentioned above. Thus, no conclusions can be drawn from comparisons between different experiments.

When the results obtained from animal groups which were handled on the same occasion are evaluated, it can be seen that pregnancy, testosterone and ethisterone administration caused an increase, while in both sexes castration, and, as a rule, oestrogen treatment elicited a decrease of the sulphate pool.

The weight gain increase and the slight increase in sulphate pool after low doses of oestradiol benzoate seen particularly in adult mice (table 4) is puzzling, but might be due to electrolyte and water retention, since oestradiol in similar doses caused markedly increased plasma-sodium levels in rats (WOOLLEY & TIMIRAS 1964).

It has previously been established that both natural sex hormones and synthetic oestrogens during the detoxication process become conjugated to ester sulphates, to a varying extent (WILLIAMS 1959; BOSTRÖM 1965). Since the marked decreases in sulphate pool and sulphate incorporation rate in the present study were found particularly after large doses of oestrogens, the possibility must be considered that these hormones when detoxicated, may have consumed a part of the available sulphate pool of the animals. Naturally the largest doses would be most likely to have depleting effects. For the most extreme case, 1 mg oestradiol benzoate per day, it can be calculated that if

the hormone had been entirely converted to a disulphate ester, about 4 μmol of sulphate would have been required. This corresponds to about one sulphate pool of a mouse (384 μg SO_4). Therefore, an important question is how fast a depleted pool can be restored in a mouse under normal circumstances. It has been shown previously that after an injection of tracer amounts of ^{35}S-sulphate practically all the tracer disappears from the blood in less than 24 hours (HERBAI 1970a). This means a total renewal of the sulphate pool during one day. Since oestradiol benzoate is gradually absorbed and since it is unlikely that it is totally converted to disulphate, it is unlikely that – barring enterohepatic circulation – even a dose of 1 mg per day would consume such a large part of the available sulphate store, that a real deficit remains during the experiment, which is done 24 hours after the last dose.

It has been reported that C_{18} steroids may undergo enterohepatic circulation (ADLERCREUTZ & LUUKKAINEN 1967; SJÖVALL 1970) and it is theoretically possible that after excretion into the intestines and desulphation, the steroids may be reabsorbed and sulphated again. This might possibly increase the sulphate consumption caused by the steroids. However, as can be seen in table 3 and 4 the sulphate pool per g body weight in the male animals is decreased to about the same extent after 10, 100 and 1000 μg oestradiol benzoate per day, which means that at least after the 2 previous doses, the amount of sulphate needed for sulphation of the hormone must be negligible as compared to the available store.

Another factor, which must be discussed is a possible competition for "active sulphate" during the sulphation reaction between oestradiol benzoate and the chondroitin sulphate chain of the cartilage. In order to study this possibility the following experiment was done in a previous work (HERBAI 1970a): Increasing amounts of phenol (up to 32 μmol) were injected intravenously into different groups of mice. The sulphate pools were determined after 30 min. using a sample of urine and the sulphation rates of cartilage were measured 4 hours after the phenol injection. The results indicated that even after very high doses of phenol (32 μmol per mouse), an amount which in other experiments would be expected to require about 16 μmol of sulphate ions from the miscible sulphate pool, the size of the sulphate pool and the rate of sulphate incorporation into the costal cartilage were completely unaffected. This means that even after the removal of 1500 μg sulphate from the sulphate pool (an amount which is 4–5 times the pool size) this is sufficiently rapid to allow unchanged sulphate utilization and an unimpaired chondroitin sulphate synthesis within a few hours. This process seems to be possible only, if a rapid renewal of consumed sulphate is maintained by an active process, the existence of which, as far as we know, has not been previously described. In this connexion a very recent report is of interest, in which it is claimed that the parathyroids and the cells secreting calcitonin

may exert a regulatory influence on sulphate homeostasis in rats (SALLIS *et al.* 1970). All these arguments make it unlikely that oestrogens even with the highest doses as given in the present experiments could have exerted any competitive influence on the size of the sulphate pool and the rate of incorporation.

In previous studies on oestrogens and sulphate incorporation into connective tissue *in vivo* the undetected lowered sulphate pools may have elicited a spurious increase in the specific activity of the injected ^{35}S-sulphate. This could have resulted in a failure to detect the sulphation inhibiting effects; it has been reported that oestrogen administration caused either unchanged or increased incorporation rates of ^{35}S-sulphate *in vivo* into different kinds of connective tissue (KOWALEWSKI 1961; WAGNER *et al.* 1968). Inhibition of sulphate incorporation after oestrogen, as demonstrated in the present study, has previously been reported by PRIEST *et al.* (1960) and NAKAMURA & MASUDA (1966) in rats which received oestradiol benzoate in doses of 333–1000 μg/day for 2–3 weeks. The sulphate pools of the animals were not checked and the lack of dose-response curves and shorter periods of hormone administration make it impossible to decide whether the effects could have been obtained with lower doses or after shorter period of treatment. Small changes in sulphation rates, as found in pregnancy and after castration in the present study (table 1) were not detected without correction for the altered sulphate pool.

The slightly elevated sulphation rate of cartilage in pregnant mice may be due to the placental "growth hormone-prolactin" described by GRUMBACH & KAPLAN (1964) who stated that this hormone elicited a weak stimulation of sulphate uptake into the cartilage of hypophysectomized rats.

Following treatment with the anabolic-androgenic agents the growth stimulating and sulphate incorporation increasing effects were not parallel (table 2). Testosterone propionate caused a significant increase in weight gain but failed to elevate the incorporation of sulphate. In contrast, ethisterone only slightly increased the weight gain, but significantly increased sulphate incorporation, at least in low doses. The weak effects of these steroids on the sulphation reaction are in agreement with the previous findings of SALMON *et al.* (1963) who found only a slight stimulation of the sulphate incorporation *in vitro* in normal rats after testosterone administration.

The dose-response curves of the present investigation show that both oestradiol benzoate and stilboestrol were able to elicit a similar and dose-dependent inhibition of growth and a decrease of sulphate incorporation in cartilage. However, all effective doses used in this study (the minimum dose which elicited a significant change was 10 μg) and those of previous reports (SILBERBERG & SILBERBERG 1941; PRIEST *et al.* 1960; NAKAMURA & MASUDA 1966) are much higher than the physiological dose range of the hormones,

being for example at least 1000 times above the minimum oestrus producing doses (0.01 μg). These massive pharmacological doses very probably influence the function of several different endocrine organs and might exert their growth-retarding actions indirectly through other endocrine glands e. g. the pituitary or thyroid. It is therefore an interesting question whether the growth inhibiting properties can be dissociated from the oestrogenic effects. For this purpose a series of substances with weak oestrogenicity have been studied under similar conditions and the results will be presented in subsequent communications.

Acknowledgements

My sincere thanks are due to Professor E. Bárány for his stimulating criticism and to Docent L. Terenius for many valuable discussions and advice. The excellent technical assistance of Mrs. B. Jansson is greatly appreciated. This work was supported by grants from the Medical Faculty of Uppsala University and from the Nordisk Insulinfond, Gentofte, Denmark.

REFERENCES

Adlercreutz, H. & T. Luukkainen: Biochemical and clinical aspects of the enterohepatic circulation of oestrogens. *Acta Endocrin.* (Kbh.) 1967, Suppl. 124, 101–140.

Berntsen, E.: Epiphyseal growth zones in oestradiol-treated rabbits. *Acta Endocrin.* (Kbh.) 1968, 57, 69–80.

Boström, H.: Sulfate conjugation and conjugated sulfates. *Scand. J. clin. Lab. Invest.* 1965, 17, Suppl. 86, 33–52.

Conney, A. H., K. Schneidman, M. Jacobson & R. Kuntzman: Drug-induced changes in steroid metabolism. *Ann. N. Y. Acad. Sci.* 1965, 123, 98–109.

Conney, A. H., M. Jacobson, W. Levin, K. Schneidman & R. Kuntzman: Decreased central depressant effect of progesterone and other steroids in rats pretreated with drugs and insecticides. *J. Pharmacol Exp. Therap.* 1966, 154, 310–318.

Conney, A. H. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev* 1967, 19, 317–366.

Dziewiatkowski, D. D, F. Bronner, N. di Ferrante & R. M. Archibald: Some aspects of the metabolism of sulphate-S^{35} and calcium-45 in the metaphyses of immature rats. Influence of β-oestradiol benzoate. *J. Biochem. Biophys. Cytol.* 1957, 3, 151–159.

Grumbach, M. M. & S. L. Kaplan. On the placental origin and purification of chorionic "growth hormone-prolactin" and its immunoassay in pregnancy. *Transact. N. Y. Acad. Sci* 1964, 27, 167–188.

Herbai, G.: A double isotope method for determination of the miscible inorganic sulphate pool of the mouse applied to in vivo studies of sulphate incorporation into costal cartilage *Acta physiol. scand.* 1970a, 80, 470–491.

Herbai, G.: Incorporation and disappearance of sulphate in different regions of mouse and rat costal cartilage *in vivo* and *in vitro* *Acta physiol. scand.* 1970b, 79, 541–551.

Herbai, G.: Effect of age, sex, starvation, hypophysectomy and growth hormone from several species on the inorganic sulphate pool and on the incorporation *in vivo* of sulphate into mouse costal cartilage. *Acta endocrin. (Kbh)* 1971a, 66, 333–351.

Herbai, G.: Effect of adrenalectomy, corticosteroids and some other anti-inflammatory agents, salazopyrin®, thyroxine and vitamin A on the exchangeable sulphate pool and on sulphate incorporation *in vivo* into costal cartilage of the mouse. *Acta pharmacol. et toxicol.* 1971b, 29, 164–176.

Hervey, E. & G. R. Hervey: The effects of progesterone on body weight in castrate rats. *J. Physiol. (Lond.)* 1965, 177, 51–52 P.

Kowalewski, K: Effect of oestradiol cyclopentylpropionate on serum and liver lipids and aortic ^{35}S-sulphated mucopolysaccharides in cockerels fed normal or atherogenic diet. *Acta endocrin. (Kbh.)* 1961, 36, 126–130.

Levin, W., R. M. Welch & A. H. Conney: Effect of phenobarbital and other drugs on metabolism and uterotropic action of estradiol-17-β and estrone. *J. Pharmacol. Exp. Therap.* 1968, 159, 362–371.

Lorenzen, I.: *Experimental arteriosclerosis. Biochemical and morphological changes induced by adrenaline and thyroxine.* Thesis. Munksgaard, Copenhagen 1963, 20–28.

Nakamura, K. & Y. Masuda: Effects of estrogens on the metabolism of acid mucopolysaccharides in rat aorta. *Biochem. Pharmacol.* 1966, 15, 803–808.

Noble, R. L.: Effect of synthetic oestrogenic substances on the body-growth and endocrine organs of the rat. *Lancet* 1938, *II*, 192–195.

Noble, R. L.: Effects of synthetic oestrogens and carcinogens when administered to rats by subcutaneous implantation of crystals or tablets. *J. Endocrin.* 1939, 1, 216–229.

Priest, R. E., R. M. Koplitz & E. P. Benditt: Estradiol reduces incorporation of radioactive sulfate into cartilage and aortas of rats. *J. exp. Med.* 1960, 112, 225–236.

Sallis, J. D., T. H. Martin, M. de Luise & R. A. Melick: Relationships of the parathyroids and calcitonin in maintaining sulphate homeostasis. *Horm. Metab. Res.* 1970, 2, 238–241.

Salmon, W. D. Jr., P. H. Bower & E. I. Thompson: Effect of protein anabolic steroids on sulfate incorporation by cartilage of male rats. *J. Lab. Clin Med.* 1963, 61, 120–128.

Silberberg, M. & R. Silberberg: Further investigations concerning the influence of estrogen on skeletal tissues. *Amer. J. Anat.* 1941, 69, 295–331.

Silberberg, M. & R. Silberberg: Steroid hormones and bone. In: *Biochemistry and physiology of bone.* Ed. G. H. Bourne, Academic Press, New York, 1956, 623–668.

Sjovall, K.: Steroid sulphates and bile acids in pregnancy. Thesis. *Opuscula Medica* (Stockholm) 1970, *Suppl. 15*, 1–23.

Swanson, H. E. & J. J. van der Verff ten Bosch: Sex differences in growth of rats, and their modification by a single injection of testosterone propionate shortly after birth. *J. Endocrin.* 1963, 26, 197–207.

Wagner, H., G. Junge-Hulsing, W. Wirth, J. Kuckulies & O. Rave: Untersuchungen über den Einfluss verschiedener Hormone und Vitamine auf den Stoffwechsel der sulfatierten Mucopolysaccharide des Bindegewebes. *Z. Rheumaforsch.* 1968, 27, *3–12*

Williams, R. T.: The metabolism of phenols. In: *Detoxication mechanisms.* Chapman & Hall Ltd., London, 1959, 278–317.

Woolley, D. E. & P. S. Timiras: Water and electrolyte alterations in plasma, brain and liver of rats after castration and sex hormone administration. Acta *Endocrin.* (Kbh.) 1964, 46, 12–24.

Acta pharmacol. et toxicol. 1971, 29, 194–208.

From the Department of Pharmacology,
Royal Danish School of Pharmacy (Professor E. Jacobsen, M.D.),
Copenhagen, Denmark

The Penetration of Quinine, Salicylic Acid, PAS, Salicyluric Acid, Barbital and Lithium across the Vitreous Barrier of the Rabbit Eye

By

Per Nellemann Sørensen

(Received June 19, 1970)

Abstract: By intravenous infusion into rabbits anaesthetized with urethane or barbital the plasma level of a number of drugs was kept constant. The drug concentrations were determined in the vitreous body, aqueous humour, lens, and plasma. The steady state distribution ratios across the blood-vitreous barrier was found to be: for quinine 1.35, salicylic acid: 0.42 and 1.0 (at plasma concentrations 150 and 900 μmol/l respectively), PAS (*p*-aminosalicylic acid): 0.65, salicyluric acid: 0.05, barbital: 0.75. The distribution rate across the blood-aqueous barrier was found to be for quinine: 0.25, salicylic acid: 0.8 and 1.0 (at plasma concentrations 150 and 900 μmol/l), salicyluric acid: 0.1, barbital: 0.95. The permeability constants for the penetration of the drugs into the vitreous body were calculated, quinine: 2.2×10^{-1}, salicylic acid: 1.4×10^{-2}, barbital: 1.2×10^{-2}, PAS: 8.5×10^{-3}, lithium: 6.9×10^{-4}, salicyluric acid: 5.3×10^{-5}, and into the aqueous humour, quinine, salicylic acid, barbital, and PAS: $1.1 - 1.5 \times 10^{-2}$, lithium: 6.2×10^{-3}, salicyluric acid: 2.9×10^{-4}, all per minute. The partly ionized drugs penetrated the vitreous barrier according to the lipid solubility of the unionized form, thus penetration across the aqueous barrier was dependent on both the lipid solubility of the unionized form and the dissociation constant. Li^+ penetrated into the vitreous body predominantly from the anterior region of the eye and the partly *ionized drugs* through the whole exchange area. The injection of *25 μl 0.9 % NaCl or liquefaction of the vitreous body with hyaluronidase gave little change in the rate of Li^+ penetration, while that of PAS was decreased by half. The ratio between the salicyluric acid concentration in the water phase of the* lens and the *aqueous humour averaged 3.6 (1.1 – 5.6), suggesting that* salicyluric acid was *transported into the lens against a concentration gradient.*

Key-words: Vitreous body – aqueous humour – permeability.

Quantitative studies have demonstrated an existence of a blood-vitreous and a blood-aqueous barrier (Davson 1956, 1962, 1969; Kinsey & Reddy 1964). The penetration across the barriers was found to be dependent on the

lipid solubility of the drug (DAVSON 1955; BLEEKER *et al.* 1968). Furthermore DAVSON & MATCHETT (1950) have demonstrated the dependence on the dissociation constant of some partially ionized compounds.

However, certain points still seem uncertain; thus BECKER (1960) has demonstrated an active transport across the barriers. The investigations on the passage through the barriers, however, have been limited to rather a few substances and a further extension with compounds of other types is desirable before any generalized statement about the passage through the eye barriers can be made. It is possible that not only the passage through the membrane, but also the diffusion through the relatively large vitreous body may be influenced by its physical state, particularly its content of hyaluronic acid which may inhibit the spread of drugs as is seen e. g. in the subcutaneous tissue.

In the present investigation 4 weak acids, 1 weak base, and 1 electrolyte were used in order to decide to what extent all transport from plasma into the vitreous body could be explained by passive mechanisms or as seems more likely by specific active mechanisms.

For the experiment we chose salicylic acid derivatives: salicylic acid, *p*-aminosalicylic acid (PAS), and salicyluric acid (glycine conjugated salicylic acid), i. e. substances with a closely related structure, but a different rate of penetration.

Barbital (diemalum NFN) and quinine were chosen because these substances are not secreted in the kidney tubules in contrast to the salicylic acid derivatives. Finally lithium was chosen in order to compare this with sodium, the penetration of which has been extensively investigated.

Materials and Methods

Male albino rabbits weighing 2.5–4.5 kg from Havdrup animal breeding were used and were maintained on a standard laboratory diet.

The chemicals and solvents used were of analytical grade, Merck.

Experimental procedure.

The rabbits were anaesthetized with urethane (1500 mg/kg body weight) or barbital sodium (diemalum NFN) (200 mg/kg body weight). Blood clotting was prevented by heparin LEO (2.500 i.u. q. 2 hrs). The animals were then tracheotomized. The blood pressure was measured from the right femoral artery, from which blood samples were also taken.

A priming dose of the drug in solution made isotonic with NaCl was administered through a marginal ear vein in less than 15 sec., and the plasma level of the drug was maintained by continuous infusion into the ear vein from a Braun infusion pump. The scheme shown in table 1 was used. In some experiments two compounds were administered together, viz. quinine and salicylic acid. The plasma concentration was kept at a constant level of the drug within the limits of ±10 %. In 4 experiments was within the limits of ±15 %.

At various times after the initial dose the eye was enucleated.

About 200 μl anterior aqueous humour was withdrawn in less than one minute by inserting a fine cannula parallel to the iris at the limbus corneae and reaching to the edge of the pupil. Immediately after this, the eye was enucleated and frozen in liquid nitrogen. The vitreous body and the lens was dissected in the frozen state without any blood contamination, and the vitreous body was divided into an anterior, a medium and a posterior part. Before the analysis the thawed vitreous gel was centrifugated in a M.S.E. ultracentrifuge at 200,000 x g for 30 min., and the clear watery supernatant was used for analysis. After weighing, the lens was homogenized in a Braun glass homogeniser with 0.1 N sulphuric acid, and centrifuged for 15 min., 7°, at 1,100 x g. In some experiments 25 μl hyaluronidase (penetrase LEO (hyaluronidasum NFN) containing 12.5 i.u.) was injected under visual control into the center of the vitreous body by means of a Hamilton 25 μl syringe 45 min. before administration of the priming dose. The vitreous bodies injected with hyaluronidase were liquid so that centrifugation of the content was not nescessary.

Analytical methods.

Samples of 0.1 ml were used from the plasma, aqueous humour, vitreous body, and lens supernatant.

Barbital was determined by the method of LOUS (1954) on a Beckman (DU) spectrophotometer. *PAS* was determined as described by RASMUSSEN (1968) on an Aminco-Bowman-spectro-fluorometer, activated at 355 nm (uncorrected).

The sample containing *salicyluric acid* was mixed with 0.4 ml 6 N sulphuric acid, 7 ml benzene and shaken vigorously mechanically for 15 min. After centrifugation 6 ml was transferred to a centrifuge tube with 2.5 ml 0.25 M borate buffer pH : 10., shaken 15 min. and if nescessary centrifuged for separation of the phases. The fluorescense of the aqueous phase was measured at 405 nm, activated at 330 nm (both uncorrected). Concentrations as low as 0.05 μg/ml were detectable with a recovery of 98–100 %. *Quinine* and *salicylic acid* were determined in the same sample. Quinine was first determined by the method of BRODIE *et al.* (1947), and salicylic acid was determined on the benzene phase by the method of CHIRIGOS & UDENFRIEND (1959) who, however, used ether as the extraction medium instead of benzene.

The recovery after benzene extraction was the same as that after ether extraction.

Since potassium and especially sodium when present in the samples greatly influences the lithium determination, the lithium concentration was measured by using blanks and standards with the same sodium and potassium content as the respective biological fluids. *Lithium* in the plasma was determined in this way as described by AMDISEN (1967).

Lithium in the vitreous body, aqueous humour, and lens was determined by the same procedure, but the sodium and potassium concentrations for plasma standards, given by AMDISEN (1967), were replaced by the sodium and potassium concentrations in the vitreous body, aqeous humour, or lens respectively. The concentrations used were for the vitreous body Na: 125 meq./l; K: 11.7 meq./l (REDDY & KINSEY 1960); for aqueous humour Na: 137.5 meq /l; K: 4.9 meq /l (REDDY & KINSEY 1960); and for the lens Na: 25 meq./l; K: 120 meq /l (VAN HEYNINGEN 1962).

Binding to plasma proteins was determined as described by AMES & SAKANOUE (1964).

The ether/saline partition coefficient was determined for drugs in the plasma concentrations obtained by shaking the drug in equal volumes of ether and an isotonic phosphate buffer, pH : 7.4 for one hour.

pH – and standard-bicarbonate-concentration were determined at intervals of about 30 min. on arterial blood, and occasionally on aqueous samples as described by GAMBINO *et al.* (1966) on a pH-meter, microelectrode G 297 (Radiometer).

The rate of entry into the vitreous body from the blood is expressed as a "permeability constant", k_{in}. When the plasma concentration is constant k_{in} is calculated from the equation derived from the Fick law of diffusion as described by DAVSON (1962, 1969).

Results

The results of the experiments are shown in fig. 1–4 and tables 1–4. As shown in fig. 1 and table 3, the drugs enter the vitreous body at widely different rates. From fig. 2 and table 3 it is seen that the drugs enter the aqueous humour more rapidly and to a less different rate than into the vitreous body. During the period of the experiments, quinine, PAS, barbital, salicyluric acid, and lithium appear to enter the vitreous body and aqueous humour at rates proportional to the concentration gradient. This was not the case for the penetration of salicylic acid into the vitreous body. A steady state distribution ratio $R_v = 1.0$ was obtained at a total plasma concentration of 900 μmol/l, whereas R_v seemed to be about 0.4 at total plasma concentration of 150 μmol/l. From fig. 2 it is seen that the salicylic acid penetration into

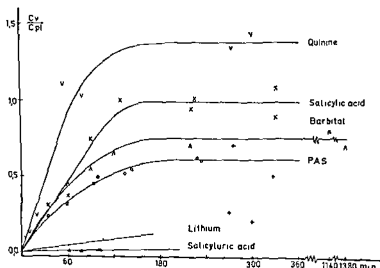


FIG. 1. Penetration of certain drugs into the vitreous body from the blood plasma. Every point represent the value for one single eye. – v – represent quinine, – x – salicylic acid at a total plasma concentration of 900 μmol/l, – + – salicylic acid at a total plasma concentration of 150 μmol/l, – Λ – barbital, – • – lithium, and – ▲ – salicyluric acid.

Table 2.

Equilibration of vitreous body, aqueous humour and lens with blood plasma. C_v, C_{aq}, and C_l denote the concentrations in the vitreous body, aqueous humour and lens, respectively, at stated intervals, after a constant concentration, C_{pl} of the drug had been obtained in the blood plasma. C_v is the mean of C_{va}, C_{vm}, and C_{vp}, which are *the concentrations in the anterior, medium, and posterior part of the vitreous body.* pH and standard bicarbonate are given as the values at half the stated intervals. In the experiments marked x or y, lithium and PAS were given simultaneously. In x experiments 25 µl hyaluronidase was injected into the vitreous body before infusion and in y experiments, 25 µl 0.9 % NaCl was injected.

A

	Rabbit no.	Time (*min.*)	C_v (*µmol/l*)	C_{aq} (*µmol/l*)	C_l (*µmol/l*)	C_{pl} (*µmol/l*)	pH *plasma*	Plasma *standard* bicarbonate (µmol/l)
Salicylic acid	1	35	66	105	33	900	7.32	21.83
	1	40	78	124	45	900	7.32	21.83
	2	*105*	*134*	*161*	*191*	772	7.32	*15.20*
	2	130	183	191	131	772	7.32	15.20
	3	220	177	192	18	824	7.35	21.33
	4	330	213	209	4	828	7.36	15.86
	4	*335*	*176*	*164*	*4*	*828*	*7.36*	*15 86*
	5	270	7.3	14.5	trace	294	7.44	21.30
	5	300	6 95	24 8	19.5	294	7 44	21.30
	6	275	10 6	15.2	66	151	7.40	22.63
	6	*325*	7.2	*14.1*	*130*	*151*	7.40	22 63
p-amino-salicylic acid	7	30	232	495	153	1405	7.35	15.52
	7	60	291	864	117	1450	7.35	15.22
	8	*105*	*461*	982	*trace*	*1706*	7.38	21.83
	8	110	477	1040	trace	1706	7.38	21.83
	9	135	503	954	203	1620	7.29	14.42
	9	145	530	954	0	1620	*7.29*	*14 42*
	10	*230*	*566*	1024	204	1513	7.30	19 01
	10	235	559	924	135	1513	7.30	19 01
	11	30 x	*77*	117	0	*914*		
	11	30 y	59	174	0	914		
	12	60 x	234	314	trace	1760		
	12	60 y	201	287	trace	1760		
	13	90 x	*121*	*268*	*21*	*2060*		
	13	90 y	135	307	7	2060		
	14	180 x	178	451	160	1188		
	14	180 y	178	440	trace	1188		
Salicyluric acid	15	60	6.3	85.6	89.9	4970	7.39	22.86
	15	75	8.1	99.9	517	4310	7.39	22.86
	16	100	11.9	65 0	162	2380	7.40	22.63
	16	*100*	*14.0*	70.1	391	2380	7.40	22.63

B

	Rab-*bit* no.	Time (min.)	C_v (μmol/l)	C_{aq} (μmol/l)	C_l (μmol/l)	C_{pl} (μmol/l)	pH plasma	Plasma standard bicarbonate (μmol/l)
Quinine	3	5	0.33	0.48	0.18	12.8	7.24	21.33
	2	10	1.02	0.77	0	31.2	7.35	16.16
	2	15	3 03	1.05	1.9	31.2	7.35	16.16
	4	20	9.01	4.26	5.49	99.7	7.39	20.85
	6	275	0.19	0 23		1.00	7.40	21.85
	6	325	0.20	0.77		1.00	7.40	21.85
	5	55	2.34	0 44	trace	6.59	7.31	15.20
	5	80	2.09	0.44		6.28	7.31	15.20
Barbital	17	60	999	1661	560	2203	7.40	23.90
	17	90	1221	2212	598	2203	7.40	23.90
	18	120	1125	1592	360	1681	7.42	23.39
	18	210	1236	1590	1100	1712	7.42	23.39
	19	*1140*	*678*	799		823	7.41	22.34
	19	1380	590	789		823	7.41	22.34
Lithium	20	60	431	2,320		11,750		
	20	60	219	2,190		11,750		
	21	60	535	2,830	trace	8,260		
	21	60	502	2,970	trace	8,260		
	22	105	137	1,100		1,610		
	22	110	94	880		1,610		
	11	30 x	62	412		1,830		
	11	30 y	98	501		1,830		
	12	60 x	253	1,190	trace	4,710		
	12	60 y	259	1,040	trace	4,710		
	13	90 x	390	1,280		5,130		
	13	90 y	283	1,310		5,130		
	14	180 x	564	2,850		3,830		
	14	180 y	568	2,690		3,830		

the aqueous humour did not show any great difference between the steady state ratios at plasma concentration 150 μmol/l and at 900 μmol/l.

The non-lipid soluble substances lithium and salicyluric acid do not pass readily into the ocular fluids (fig. 1–2, table 2 & 3).

Of the drugs partly ionized at pH 7.4, the lipid soluble quinine readily enters into both the vitreous body and the aqueous humour. Salicylic ... enters the vitreous body more rapidly than barbital.

Vitreous body equilibrium with adjacent tissues.

Table 4 shows the relative concentrations of the drugs in the three sections of the vitreous body. It is seen that *salicylic acid* has the lowest concentration in the middle of the vitreous body, while a less pronounced trend is seen with *quinine, barbital,* and *PAS.*

Furthermore the concentration of these drugs in the posterior part is equal to the concentration in the anterior part. *Lithium* shows decreasing concentration in the anterio-posterior direction. The *salicyluric acid* concentration gradient appears to rise in the anterio-posterior direction. From table 2 it is seen that the ratio between the salicyluric acid concentration in the waterphase of the lens and the anterior aqueous humour averaged 3.6 (1.1–5.6.)

Table 3.

The rates of entry of certain drugs from the plasma into the vitreous body and into the anterior aqueous humour of the rabbit.

The permeability constants are calculated from the data in table 2A and B.

	Per cent bound to plasma proteins		Calculated per cent unionized at pH 7.39	Determined ether/water partition coefficient at pH 7.4	*Heptane/water partition coefficient of the unionized drug	Permeability constant k_{in} (min.$^{-1}$)	
	Concentration µmol/l	%				Vitreous	Aqueous
Quinine	4 10 215	83 70 60	9.09	5.8	---	2.2×10^{-1}	
Salicylic acid	150 900	90 76	4×10^{-3}	1.5×10^{-2}	1.2×10^{-1}	1.4×10^{-2}	1.1–1.5 $\times 10^{-2}$ min.$^{-1}$
Barbital	1500	< 2	55.7	8.0×10^{-2}	1×10^{-3}	1.2×10^{-2}	
PAS	1500	40	2×10^{-2}	3.8×10^{-3}	---	8.5×10^{-3}	
Lithium	4 meq./l	< 4	---	---	---	6.9×10^{-4}	6.2×10^{-3}
Salicyluric acid	3000	2	2×10^{-2}	5.5×10^{-4}	---	5.3×10^{-5}	2.9×10^{-4}

* According to Hogben *et al.* (1959).

Table 4.

The relative concentrations of the drugs in the three sections of the vitreous body after constant levels of the drug have been maintained, and when $C_{va} = 1.00$ = the drug concentration in the anterior part. C_{vm} and C_{vp} denote the drug concentrations in the medium and the posterior part respectively.

	C_{vm}/C_{va}	Number of eyes	C_{vp}/C_{va}	Number of eyes
Quinine	0.926	7	1.097	6
Salicylic acid	0.925	11	1.082	11
Barbital	0.912	4	0.962	6
PAS	0.973	8	1.074	8
PAS*	0.956	7	0.918	7
Salicyluric acid	1.125	4	1.553	4
Lithium	0.560	5	0.413	6
Lithium*	0.727	8	0.626	7

* PAS and lithium were administered simultaneously after which the injection of 25 µl hyaluronidase or of 0.9 % NaCl into the vitreous body was performed.

Rate of penetration after hyaluronidase treatment of the vitreous body.

Fig. 3 shows little difference in the lithium penetration into the vitreous body between the eyes injected with 25 µl hyaluronidase and the corresponding eyes into which 25 µl 0.9 % NaCl was injected. However, for further control PAS was injected intravenously simultaneously with lithium, and as is seen in fig. 4, the PAS penetration into the injected eyes is only at half the rate of the PAS penetration into the uninjected eyes irrespective of the administration of hyaluronidase.

Discussion

pH gradients are found across the blood-vitreous and the blood-aqueous barriers. The pH in the vitreous body, pH_v, is acid (7.30), and the pH in the aqueous humour, pH_{aq}, is alkaline (7.55) in relation to the pH of the plasma (Constant 1961; Davson 1962, 1969).

If the ocular barriers are selectively permeable to the undissociated molecules, the steady state distribution ratio can be expressed by the pH partition hypothesis (Schanker 1964)

$$R_v = \frac{1 + 10^{\pm(pH_v - pKa)}}{1 + 10^{\pm(pH_{pl} - pKa)}}$$

where (+) is used for acids, (−) for bases and pKa is the dissociation constant. pH_{pl} is pH in plasma.

The theoretical distribution ratios across the ocular barriers were calculated from pH measurements. At the start of the experiment, pH_{pl} was found to be 7.39 (7.387 ± 0.012, S. E. M. for n = 15).

None of the acids were in excess in the aqueous humour. This observation can not be explained by a decreasing plasma level of the drug or by an increasing plasma pH during the experiment.

The acids barbital, PAS, and salicyluric acid did not reach the steady state ratios 0.94, 0.79 and 0.79, respectively, as predicted from the pH-partition hypothesis, and entered the vitreous body rather slowly. On the other hand salicylic acid showed a R_v = 1.0 (at 900 μmol/l) which is higher than the calculated R_v = 0.79. This may be explained by the steadily decreasing pH (0.03 pH unit per hour) during the experiment due to a metabolic acidosis as seen from the decreasing standard bicarbonate concentration.

The permeability of weak acids and bases across the blood-vitreous barrier depends on their molecular configuration. The lipid solubility of the unionized part of the drug appears to overshadow the effect of the dissociation constant. This appears from the difference in penetration rate of barbital and salicylic acid. The two drugs have about the same lipid solubility at pH 7.4, but the weaker acid, barbital, which has 10^{-4} times as great a fraction of the drug unionized at pH 7.39 as has salicylic acid, penetrated the blood-vitreous barrier more slowly because of the lower lipid solubility of the unionized drug (table 3).

If the unionized compounds have the same lipid solubility, the dissociation constant will determine the rate of penetration into the ocular fluids, as demonstrated with sulphonamides by DAVSON & MATCHETT (1960).

The penetration rate into the aqueous humour is dependent on the lipid solubility at pH 7.4, and accordingly both the dissociation constant and the lipid solubility of the unionized drug are important.

Only the most rapidly penetrating substance, quinine, with a penetration rate of $k_{in} = 2.2 \times 10^{-2}$ (min.$^{-1}$) for the permeability across the blood-vitreous barrier, produces a steady state ratio in accordance with the pH partition hypothesis. The fact that the weak acids deviate from the hypothesis may be explained in two ways.

One possibility is that the drug penetrates the barrier too slowly into the ocular fluids, so that a true equilibrium is never reached. This holds especially for the vitreous body in which no significant circulation is found, as seen from the low drug concentration in the middle of the vitreous (own observation) and MAURICE (1957).

Another possibility is that an active mechanism transporting certain weak acids out of the ocular fluids may be present; such a mechanism has been

demonstrated *in vivo* for iodopyracet (diodonum NFN) (FORBES & BECKER 1960; BECKER & FORBES 1961). CONSTANT (1968) showed that the penetration rate of DMO (5.5-dimethyl-2.4-oxazolidinedione) across the vitreous barrier increased with increasing plasma concentration. Thus the steady state ratio R_v for salicylic acid was only about half the value (0.42) at 0.15 mM than at 0.9 mM (1.0).

This effect on the aqueous humour concentration was slight and was not seen in CONSTANT's (1968) experiments.

The drugs found to be transported actively out of the vitreous body are also secreted into the proximal kidney tubules (BECKER & FORBES 1961) and are actively transported out of the cerebrospinal fluid (DAVSON 1967).

One of these drugs, iodopyracet, is accumulated *in vitro* against a concentration gradient in a ciliary body-iris preparation (BECKER 1960; FORBES & BECKER 1961); and it has therefore been suggested that the ciliary body is an anatomical site for the transport out of the eye of weak acids.

The results presented here indicate that the lens is partly responsible for the low vitreous body concentration of salicyluric acid. As shown in table 2, the rabbit lens was able to accumulate salicyluric acid against a concentration gradient *in vivo*. The salicyluric acid accumulation was found to be at least 0.9×10^{-3} μmol/min. per lens, which is in agreement with the maximum turn-over of 1.0×10^{-2} μmol/min. found by FORBES & BECKER (1960), when iodopyracet was injected into the vitreous body.

PAS also belongs to this group of drugs and gave a low R_v value despite a high plasma concentration. However its poor penetration rate can be explained by its low lipid solubility.

Lithium.

The lipid soluble drugs penetrated the blood-vitreous barrier through the whole exchange area (table 4), but lithium was found to penetrate the blood-vitreous barrier predominantly from the anterior part of the eye. This is also true for sodium, potassium, and phosphate (DAVSON 1962). The rate of penetration from the blood into the middle of the vitreous body was $k_{in} = 6.9 \times 10^{-4}$ (min.$^{-1}$), and from the blood into the anterior aqueous humour $k_{in} = 6.2 \times 10^{-3}$ (min.$^{-1}$). Both penetration rates are calculated on the assumption that lithium does not decrease the secretion of aqueous humour, as suggested by BERGGREN (1967); and that R_v and R_{aq} attain the Gibbs-Donnan equalibration ratio 0.96 (DAVSON 1955, 1962). R_{aq} appeared to reach this value despite the fact that the aqueous humour is about 10 mV positive to the blood (DAVSON 1962), and thus tends to lower the R_v reaches the value of 0.96 for as long as 24 hrs, provided that tl penetration does not decrease with time.

Lithium has chemical properties s' ... to sodium. The rate

of Na^+ into the vitreous body has been found by FRIEDENWALD & BECKER (1955) and MAURICE (1957) to be $k_{in} = 1.55 \times 10^{-3}$ (min.$^{-1}$) and $k_{in} = 1.56 \times 10^{-3}$ (min.$^{-1}$) $\pm$ 0.06 (S. E. M.), respectively. The ratio between the penetration rates into the vitreous body for lithium and sodium is 0.44.

The rate of sodium penetration across the blood-aqueous barrier was found by FRIEDENWALD & BECKER (1955) to be $k_{in} = 1.4 \times 10^{-2}$ (min.$^{-1}$) for the permeability by filtration into the anterior aqueous humour. If lithium penetrates into the aqueous humour by filtration, the ratio between the lithium and sodium penetration rates into the anterior aqueous humour will be 0.44.

The difference in penetration between sodium and lithium may partly be due to a greater hydration of the lithium ion; the ion radii measured by ion motility are for lithium 2.31 A, and for sodium 1.78 A (USSING 1960). Since the ratio between the radii is 0.77, a difference in ion motility cannot therefore be the only explanation for the decreased ocular penetration of lithium in relation to sodium.

Other factors may be a lithium induced decrease of aqueous humour secretion as suggested by BERGGREN (1967), or that the medium radius size of the water bridges in the blood-ocular barrier is about equal to the radius of the lithium ion, and thus offers a higher resistance to the permeability for lithium than for sodium.

The latter hypothesis is the more probable since salicyluric acid with a molecular weight of 181 hardly enters the ocular fluids.

The results found with Li^+ penetration agree with smaller permeability of Li^+ as compared with Na^+ through biological membranes as found by SOLOMON (1952), MAIZELS (1951), and ARMETT & RITCHIE (1963).

Other studies show about the same permeability for sodium as for lithium (USSING 1960; MOORE *et al.* 1966; MEEVES 1966).

Hyaluronidase treatment of the vitreous body.

The lithium permeability across the blood-vitreous barrier, when 25 µl hyaluronidase was injected into the vitreous body did not show any major difference as compared to the lithium penetration into the vitreous body of the other eye, in which 25 µl 0.9 % NaCl was injected. However, it was not possible to decide whether the hyaluronic acid present in the vitreous body offers a resistance to diffusion because of the large hydrodynamic volume of hyaluronic acid (BALAZS 1961).

For sodium, MAURICE (1957) found, that the penetration of this ion into the aqueous humour from the vitreous body could be explained on the basis of free diffusion.

The disturbing errors in this experiment were the ill-defined "hyaluronidase-like" effect of 0.9 % NaCl injected in the connective tissue (SECHER-

HANSEN *et al.* 1968), and a possible rise in intraocular pressure after injection of 25 μl fluid into the vitreous body, resulting in a decreased choriodal and retinal blood flow and a decreased aqueous humour secretion (MAURICE 1957; BERGGREN 1967).

Thus the simultaneous administration of PAS showed that PAS penetrated into the aqueous and vitreous humours at a rate, which was only half of that observed, when no fluid was injected into the vitreous. However, the decreased PAS penetration may also have been due to a lithium induced decrease of the ocular blood flow. The plasma concentration of lithium resulted in a fall in blood pressure of about 30–40 mmHg, and a reduced aqueous humour secretion may well be secondary to the decrease in blood pressure.

REFERENCES

Amdisen, A.: Serum lithium determinations for clinical use. *Scand. J. Lab. Clin. Invest.* 1967, **20**, 104–108.

Ames, A. & M. Sakanoue: A new method for preparing a plasma ultrafiltrate. Distribution of 5 electrolytes. *J. Lab. Clin. Med.* 1964, **64**, 168–176.

Armett, C. J. & J. M. Ritchie: On the permeability of mammalian non-myelinated fibres to sodium and to lithium ions. *J. Physiol. (Lond.)* 1963, **165**, 130–140.

Balazs, E. A.: Molecular morphology of the vitreous body. In: *The structure of the eye.* Ed: G. K. Smelser. Proc. of the symp. April 1960 during the VII int. anat. congress. Academic Press, New York, 1961.

Becker, B.: The transport of organic anions by the rabbit eye. I. In vitro iodopyracet (diodrast) accumulation by ciliary body-iris preparations. *Amer. J. Ophthal.* 1960, **50** (Pt. 2), 862–867.

Becker, B. & M. Forbes: Iodopyracet (diodrast) transport by the rabbit eye. *Amer. J. Physiol.* 1961, **200**, 461–464.

Berggren, L.: The intraocular pressure in rabbits after lithium administration with comments on pressure effects of injections into the vitreous. *Acta opthal.* 1967, **45**, 229–238.

Bleeker, G. M., N. J. van Haeringen, E. R. Maas & E. Glasius: Selective properties of the vitreous barrier. *Exp. Eye Res.* 1968, **7**, 37–46.

Brodie, B B., S. Udenfriend, W. Dill & G. Downing: The estimation of basic organic compounds in biologic material. II Estimation of fluorescent compounds. *J. biol. Chem.* 1947, **168**, 311–318.

Chirigos, M. A. & S Udenfriend. A simple fluorometric procedure for determining salicylic acid in biologic tissues. *J. Lab. Clin. Med.* 1959, **54**, 769–772.

Constant, M.: The distribution of 5.5–dimethyl-2.4–oxazolidinedione (DMO) in ocular and cerebrospinal fluids. *Amer. J. Ophthal.* 1961, **51**, (Pt. 2), 969–976.

Constant, M.: Distribution of 5.5–dimethyl-2.4–oxazolidinedione (DMO) in intraocular fluids. V. Effect of plasma level, probenicid, and dichlorphenamide on time course. *Invest. Ophthal.* 1968, **7**, 335–346.

Davson, H.: A comparative study of the aqueous humour and cerebrospinal fluid in the rabbit *J. Physiol.* 1955, **129**, 111–133.

Davson, H.: *Physiology of the ocular and cerebrospinal fluids.* Churchill, London, 1956.

Davson, H.: *Physiology of the cerebrospinal fluid*. Churchill, London, 1967.
Davson, H.: The intraocular fluids. In: *The eye*. Vol. I, Ed: H. Davson. Academic Press, London, 67–145, 1962. Edition 2: pp. 67–186, 1969.
Davson, H. & P. A. Matchett: The penetration of some sulphonamides into the intraocular fluids of the cat and rabbit. *J. Physiol.* 1950, **110**, 416–421.
Forbes, M. & B. Becker: The transport of organic anions by the rabbit eye. II. In vivo transport of iodopyracet (diodrast). *Amer. J. Opthal.* 1960, **50**, 867–875.
Forbes, M. & B. Becker: The transport of organic anions by the rabbit ciliary body. IV. Acetazolamide and rate of aqueous flow. *Amer. J. Ophthal.* 1961, **51** (Pt. 2), 1047–1051.
Friedenwald, J. S. & B. Becker: Aqueous humour dynamics. Theoretical considerations. *Amer. J. Ophthal.* 1956, **41**, 383–398, and *Arch. Ophthal.* (N. Y.) 1955, **54**, 799–815.
Gambino, S. R., P. Astrup, R. G. Bates, E. J. M. Campbell, F. P. Chinard, G. G. Nahas, O. Siggaard-Andersen & R. Winthers: Report of the ad hoc committee on acid-base methology. *Amer. J. Clin. Path.* 1966, **46**, 376–381.
van Heyningen, R.: The lens. In: *The eye*. Vol. I. Ed. H. Davson. Academic Press, London, 213–287, 1962.
Hogben, C. A. M., D. J. Tocco, B. B. Brodie & L. S. Schanker: On the mechanism of intestinal absorption of drugs. *J. Pharmacol. Exp. Ther.* 1959, **125**, 275–282.
Kinsey, V. E. & D. V. N. Reddy: Chemistry and dynamics of aqueous humour. In: *The rabbit in eye research*. Ed.: J. M. Prince, Ch. T. Thomas, Springfield, 218–319, 1964.
Lous, P.: A spectrophotometric method for determining barbituric acids in body fluids *Acta pharmacol. et toxicol.* 1954, **10**, 134–146.
Maizels, M.: Factors in the active transport of cations. *J. Physiol.* 1951, **112**, 59–83.
Maurice, D. M.: The exchange of sodium between the vitreous body and the blood and aqueous humour. *J. Physiol.* 1957, **137**, 110–125.
Meeves, H.: Experiments on internally perfused squid axons. *Ann. N. Y. Acad. Sci.* 1966, **137** (Art. 2) 807–817.
Moore, J. W., N. Anderson, M. Blaustein & M. Takata: Alkali cation selectivity of squid axon membrane. *Ann. N. Y. Acad. Sci.* 1966, **137** (Art. 2), 818–829.
Rasmussen, S.: Correlation between in vitro and in vivo disintegration times of enteric-coated tablets. *J. Pharm. Sci.* 1968, **57**, 1360–1363.
Reddy, D. V. N. & V. E. Kinsey: Composition of the vitreous homour *Arch. Ophthal.* (N.Y.) 1960, **63**, 167–172.
Schanker, L. S.: Physiological transport of drugs. In: *Advances in drug research* Vol. I. Ed: N. J. Harper & A. B. Simmonds. Academic Press, London, 71–106, 1964.
Secher Hansen, E., H. Langgård & J. Schou: Studies on the subcutaneous absorption in mice IV: Absorption of carbohydrates with different molecular weights from connective tissue. *Acta pharmacol. et toxicol.* 1968, **26**, 9–14.
Solomon, A. K.: The permeability of the human erythrocyte to sodium and potassium. *J. gen. physiol.* 1952, **36**, 57–110.
Ussing, H. H.: *The alkali metal ions in biology*. I. The alkali metal ions in isolated systems and tissues. In: *Handbuch der experimentellen Pharmakologie*. Erg. Werk. Bd. 13. Springer-Verlag, Berlin, 1–195, 1960.

Acta pharmacol. et toxicol. 1971, 29, 209–225.

From the Psychiatric Research Center, Ulleråker Hospital,
University of Uppsala, Uppsala, Sweden

Effects of Acute and Chronic Amphetamine Intoxication on Brain Catecholamines in the Guinea Pig*

By

Tommy Lewander

(Received September 9, 1970)

Abstract: The effects of amphetamine on central and peripheral catecholamines have been studied in guinea pigs, since in this species, unlike several others, amphetamine is not metabolized by *p*-hydroxylation. Twenty mg/kg of dl-amphetamine-sulphate given intraperitoneally caused a 40 % decrease in brain and heart noradrenaline, a 13 % decrease in brain dopamine and a 60 % decrease in homovanillic acid in the caudate nucleus, four hours after its administration. The changes in tissue catecholamine levels and the increase in motor activity followed the time-course of the amphetamine concentrations both in the brain and plasma. After chronic administration of amphetamine at 12 hourly intervals for 7 or 18 days, there was a further decrease in brain and heart catecholamine and homovanillic acid levels. A 4-fold increase in the 3-O-methylated metabolites of noradrenaline and dopamine in brain after the administration of amphetamine to guinea pigs pretreated with nialamide and an increase in the urinary excretion of NA (13-fold) and adrenaline (3-fold) provided evidence for an amphetamine induced release of central and peripheral catecholamines as has previously been reported in rats and cats. Amphetamine disappeared from brain and plasma with an apparent half-life of 2.5-3.1 hours. Only amphetamine and hippuric acid were recovered in the urine after the administration of radioactively labelled amphetamine. No *p*- or β-hydroxylated metabolites of amphetamine were present in the brain or heart tissues in the guinea pig. The results show that acute and chronic amphetamine administration causes changes in endogenous catecholamines in guinea pigs similar to those found previously in rats in spite of differences in the metabolism of amphetamine between the two species.

Key-words: Amphetamine – catecholamines – brain – guinea-pig.

The central stimulant effects of amphetamine is dependent on an unimpaired synthesis of brain noradrenaline (NA) and dopamine (DA) in rats (QUINTON & HALLIWELL 1963; WEISSMAN & KOE 1965; RANDRUP & MUNK-

*) Part of the present results have been given as a preliminary communication at the XXVI Scandinavian Pharmacological Meeting, Oslo 1970. Published in *Acta pharmacol. et toxicol.* 1970, 28, Suppl. 1, 62.

VAD 1966; DINGELL *et al.* 1967) and cats (HANSSON 1966). An increase in the levels of the 3-*O*-methylated metabolites of NA and DA, normetanephrine (NM) and methoxytyramine (MTA) respectively, in the rat brain after amphetamine (GLOWINSKI & AXELROD 1965; CARLSSON *et al.* 1965 & 1966; GLOWINSKI *et al.* 1966; GUNNE & LEWANDER 1967 & 1968; SCHEEL-KRÜGER & RANDRUP 1967) has supported the hypothesis that amphetamine acts through release of brain catecholamines (CA).

In rats *p*-hydroxynorephedrine is formed from amphetamine and is incorporated into CA storage granules as a false transmitter, a fact which seems to explain the long-lasting depletion of tissue NA levels after acute and chronic amphetamine administration (GOLDSTEIN & ANAGNOSTE 1965; LEWANDER 1968a, 1970 & 1971a; GROPPETTI & COSTA 1969a; COSTA & GROPPETTI 1970; BRODIE *et al.* 1970).

Most animal studies on amphetamine have been performed on the rat as the experimental animal. Unlike rats, guinea pigs do not metabolize amphetamine through *p*-hydroxylation (DRING *et al.* 1970). It was, therefore, thought of interest to study the relation between the elimination rate and metabolism of amphetamine and its effects in this species on CA. In addition changes in the DA metabolite, homovanillic acid (HVA), were followed in the guinea pig brain after amphetamine administration.

Material and Methods

About 150 male, mottled guinea pigs with a body-weight of 250–300 g were used. Unless otherwise stated, the animals were housed in individual cages during the experiments. Pellets, hay and water were provided *ad libitum*. Racemic amphetamine sulphate was administered intraperitoneally as described in Results. Doses refer to the sulphate. D,L-amphetamine-7-^{14}C sulphate, obtained from CEA, Saclay, Gif-sur-Yvette, France, with a radiochemical purity of >99 % after ion-exchange chromatography (LEWANDER 1971a) was diluted with suitable amounts of unlabelled drug and administered intraperitoneally.

Urine from individual metabolism cages was collected for 16 hours after the injection of the labelled or unlabelled drug and immediately frozen in beakers placed on dry ice in a thermos flask.

The motor activity was measured with an ANIMEX Activity Meter (AB Farad Electronics, Hägersten, Sweden), which records both locomotion and movements on the spot. Behavioural observations were made through a one way screen.

The animals were killed by decapitation under light chloroform anaesthesia. Blood from the neck wound was collected in heparinized tubes. The brains were removed and dissected into the following parts: a) brain stem (0.8–1.1 g) including medulla, pons, mesencephalon and diencephalon, b) telencephalon (1.8–2.1 g) including the remaining parts of the brain except the cerebellum, the hypophysis and the epiphysis, which were discarded; c) in some experiments the caudate nuclei (0.14–0.20 g) were freed and used for determination of HVA. The brain (except the caudate nuclei, see below) or heart tissues were homogenized in 15 ml 0.4 M perchloric acid and centrifuged for 10 min.

at 10,000 × g. The supernatants were adjusted to pH 4 0 with 2 M potassium hydroxide and recentrifuged for removal of the potassium perchlorate precipitate. This second supernatant was used for the following determinations.

Amphetamine in 4 ml of plasma and 2 ml of brain extract was analyzed by gas-liquid chromatography according to ÄNGGÅRD *et al.* (1970). Labelled amphetamine and possibly labelled basic metabolites of amphetamine (norephedrine, *p*-hydroxynorephedrine, *p*-hydroxyamphetamine) in the brain and heart were separated by ion-exchange chromatography and subsequent TLC as described previously (LEWANDER 1971a). The radioactivity in the urine after the administration of labelled amphetamine was analyzed by paper chromatography (ELLISON *et al.* 1966). NA and DA in tissue extracts were adsorbed on alumina and determined fluorimetrically (Aminco-Bowman spectrophotofluorimeter), (CHANG 1964; CARLSSON & WALDECK 1958). In some experiments NM and MTA in the effluent and washings from the alumina columns were separated on Amberlite CG 120, type II, ion-exchange columns (0.4 × 5 cm, Na^+-form). Elution was performed with 1 M hydrochloric acid after washing the columns twice with 10 ml of water and 2 ml of 1 M hydrochloric acid. NM in the first 14 ml portion of the eluate was determined fluorimetrically according to HÄGGENDAL (1962). MTA was eluted in a 12 ml portion of 4 M hydrochloric acid, which was evaporated to dryness. The

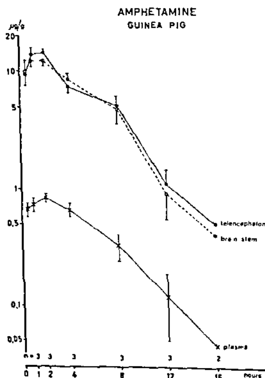


Fig 1. Amphetamine levels in µg/g (mean ± S. E. M.) in the brain stem, the telencephalon and the plasma of guinea pigs at different time intervals after the injection of 20 mg/kg intraperitoneally of dl-amphetamine-SO_4.

residue was dissolved in 5 ml of M potassium chloride and MTA was determined according to CARLSSON & WALDECK 1964. Recoveries were: NA: 88 %, DA: 90 %, NM 75 % and MTA: 80 % respectively.

Urinary adrenaline (A) and NA was determined according to EULER & LISHAJKO (1961).

The caudate nuclei from one guinea pig were homogenized in 1.0 ml of 0.4 M perchloric acid and diluted to 10 ml with redistilled water. HVA in the extract, adjusted to pH 6.5 with KOH, was isolated by ion-exchange chromatography (Dowex 1-X2) and assayed fluorimetrically, essentially according to KORF *et al.* (1970). Omission of Zn^{2+}-ion in the preparation of the fluorophor was necessary in order to get linearity between the amounts of HVA and the fluorescence readings. After preparation of the fluorophor and during fluorimetry the samples were kept at a constant temperature of +16° (or less) in a water bath. At higher temperatures the fluorescence intensity of the fluorophor became variable. Recovery of HVA was 88 %.

All values of amphetamine and CA are given as μg ± S. E. M. of their respective bases per g tissue or for 16 hrs (urinary excretion).

Student's t-test was used for statistical comparisons.

Results

Disappearance rate of amphetamine in the brain and plasma.

The levels of amphetamine, determined by GLC, in the telencephalon, brain stem and plasma at different intervals after 20 mg/kg of amphetamine intraperitoneally are shown in fig. 1. Peak levels were found at 2 hours after the amphetamine injection. In a few samples analysed at 24 hrs, amphetamine was still present. Between 2 and 16 hrs the elimination of amphetamine appeared to be exponential with a half-life of 2.8 hrs for telencephalon, 2.5 hrs for brain stem and 3.1 hrs for plasma. The $t_{1/2}$-times were determined after calculation of the regression-lines according to the method of least squares. The mean ratio between telencephalic and brain stem concentrations

Table 1.

Urinary excretion of radioactively labelled amphetamine and hippuric acid in three guinea pigs during 16 hours after administration of 5 μci of dl-amphetamine-SO_4, 20 mg/kg intraperitoneally.

Guinea pig no.	Radioactivity in urine/16 hrs % of inj. dose	Amphetamine %	Hippuric acid %
1	47	37	63
2	43	77	23
3	48	52	48
Mean	46	55	45

was 1.06, but the amphetamine levels did not differ significantly between the two parts of the brain. A constant ratio between brain and plasma of 15–16 was found during the first 8 hours after amphetamine administration. At later times the ratio was about 8.

Absence of basic metabolites of amphetamine in the urine and tissues.
The urinary excretion of radioactivity and the pattern of amphetamine metabolites was studied in 3 guinea pigs (table 1). About 46 % of the injected dose was recovered in the urine during the first 16 hours. Of the excreted radioactivity about 55 % coincided with authentic amphetamine and 45 % with hippuric acid on paper chromatography. There were wide individual variations in the distribution between the two metabolites of amphetamine. No radioactivity was found with the Rf-values of *p*-hydroxyamphetamine, *p*-hydroxynorephedrine or norephedrine. The pH of the urine was 7.5–8.5.

^{14}C-labelled amphetamine (20 mg/kg intraperitoneally) with a specific activity of 8000 cpm/μg was administered to three guinea pigs, which were killed after 2–12 hours. The brain and heart extracts were analyzed for basic metabolites of amphetamine (see Methods). Only labelled amphetamine was found in these samples; no radioactivity co-chromatographed with authentic *p*-hydroxyamphetamine, *p*-hydroxynorephedrine or norephedrine.

Time-course of the amphetamine induced increase in motor activity and stereotype behaviour.
The motor activity measured as counts per hour in 3 guinea pigs housed together was plotted against time in a semilogarithmic system after the amphet-

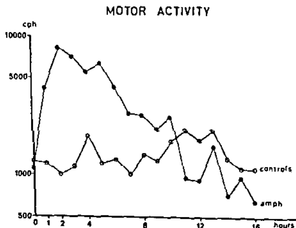


Fig 2. Changes in motor activity after 20 mg/kg intraperitoneally of dl-amphetamine-SO_4 (amph.) Each point represents the accumulated counts per hour (cph) after the injection of amphetamine or saline into three guinea pigs housed together.

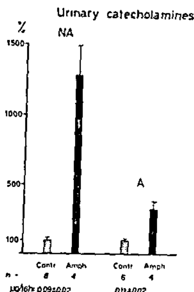


Fig. 3. Effect of 20 mg/kg dl-amphetamine-SO_4 intraperitoneally on the urinary excretion of noradrenaline (NA) and adrenaline (A). Columns represent excretion values as percentages (mean ± S. E. M.) of the control levels. The urine was collected during 16 hours after the administration of amphetamine or saline.

amine injection (fig. 2). It is shown that the changes in motor activity have about the same time course as that of the tissue amphetamine concentrations. The half-time of amphetamine action based on the decreasing activity counts between 2–12 hours was about 3.5 hrs.

Stereotype behavior consisting in intense sniffing, licking and gnawing was regularly observed for about 10–12 hours after 20 mg/kg of amphetamine given intraperitoneally. It was found necessary to make the behavioral observations in a quiet room through a one-way screen, since even the slightest disturbance abolished all signs of amphetamine induced behavioral changes.

Urinary excretion of adrenaline (A) and NA.
The urinary excretion of A and NA was measured during 16 hrs after 20 mg/kg amphetamine given intraperitoneally. As shown in fig 3, there was a 3-fold increase in A excretion while the urinary NA was increased by a factor of 13. The excretion values after saline were 0.11 µg per 16 hrs for A and 0.09 µg per 16 hrs for NA.

Time-course of changes in tissue CA levels after amphetamine.
In fig. 4 the changes in the levels of NA in the brain stem, and NA and DA in telencephalon after 20 mg/kg of amphetamine given intraperitoneally are shown.

There was a decrease in both telencephalic and brain stem NA to about 60 % of the control level at 4–8 hours after the amphetamine injection, with a slow return towards initial values at 24 hours. The changes in heart NA levels were similar to those found in the brain (data not shown).

The DA in telencephalon was decreased to 87 % of the control level at four hours after amphetamine administration, but was not changed at any other time.

Brain levels of NM and MTA after amphetamine.

The concentrations of NM in the brain stem and telencephalon and MTA in the telencephalon were measured at different times (1–8 hrs) after amphetamine administration. In all instances as well as in the control animals the levels were below the sensitivity of the methods used. Therefore, NM and MTA levels were determined after the inhibition of monoamine oxidase with nialamide given 20 hours previously. As shown in table 2, increased CA levels and measurable amounts of NM and MTA were obtained in the brain sections studied in nialamide pretreated animals. The administration of amphetamine after nialamide was followed by a 4-fold increase in the brain stem and telencephalic NM and telencephalic MTA.

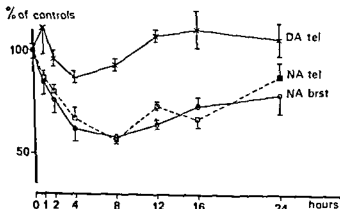


Fig. 4. Changes in the noradrenaline content of the brain stem (NA_{Brst}) and the telencephalon (NA_{Tel}) and in telencephalic dopamine (DA_{Tel}) at intervals after 20 mg/kg intraperitoneally of dl-amphetamine-SO_4. Values are expressed as percentages (mean ± S. E. M., n = 3–6) of control levels, which were: $NA_{Brst} = 0.45 \pm 0.02$ µg/g (n = 12), $NA_{Tel} = 0.15 \pm 0.004$ µg/g (n = 12), $DA_{Tel} = 0.74 \pm 0.02$ µg/g (n = 6). Open symbols or + indicate statistically significant differences ($P < 0.05$–0.001) from control values.

Table 2.

Effects of amphetamine on the levels (μg/g ± S. E. M.) of noradrenaline (NA), normetanephrine (NM), dopamine (DA) and methoxytyramine (MTA) in the brain stem and the telencephalon of control and nialamide pretreated guinea pigs. Figures in brackets = numbers of observations.

Treatment	Brain stem		Telencephalon			
	NA	NM	NA	NM	DA	MTA
Saline (9–12)[a]	0.45 ± 0.02	< 0.04	0.15 ± 0.004	< 0.04	0.85 ± 0.06	< 0.03
Amphetamine[a] 20 mg/kg i. p. 1 hr (3–7)	0.38 ± 0.03	< 0.04	0.13 ± 0.005	< 0.04	0.82 ± 0.10	< 0.03
Nialamide 100 mg/kg i. p. 20 hrs (5)	0.86 ± 0.02[b]	0.093 ± 0.009	0.36 ± 0.01[b]	0.068 ± 0.005	1.44 ± 0.07[b]	0.080 ± 0.013
Nialamide + Amphetamine (5)	0.68 ± 0.02[c]	0.44 ± 0.05[c]	0.33 ± 0.02	0.19 ± 0.02[c]	1.46 ± 0.05	0.32 ± 0.01[c]

[a] Data from fig. 3.
[b] Different from saline: $P < 0.001$.
[c] Different from nialamide: $P < 0.001$.

Table 3.

Tissue content of noradrenaline (NA), dopamine (DA), homovallinic acid (HVA) and amphetamine (Amph.) at 4 hours after 1, and after the 14th and the 36th injection of dl-amphetamine-SO_4 given intraperitoneally at 12 hourly intervals.

Treatment	Telencephalon					Brain stem			Caudate nucl.		Heart	
	NA µg/g	%	DA µg/g	%	Amph. µg/g	NA µg/g	%	Amph. µg/g	HVA µg/g	%	NA µg/g	%
Saline (n = 5)	0.16 ± 0.01	100	1.04 ± 0.04	100	–	0.49 ± 0.02	100	–	1.53 ± 0.10	100[c]	0.80 ± 0.06	100
Amph. 20 mg/kg × 1 (n = 5)	0.10 ± 0.004[a]	63	0.89 ± 0.02	86	10.9 ± 1.28	0.31 ± 0.03[a]	63	8.9 ± 0.84	0.64 ± 0.18[a]	42[c]	0.52 ± 0.03[a]	65
Saline (n = 4)	0.13 ± 0.007	100	–	–	–	0.31 ± 0.03	100	–	1.62 ± 0.26	100	–	–
Amph. 20 mg/kg × 2 for 7 days (n = 5)	0.03 ± 0.005[a]	23	–	–	17.7 ± 1.93[b]	0.15 ± 0.01[a]	48	17.1 ± 2.04[a]	0.36 ± 0.13[a]	22	–	–
Saline (n = 4)	0.17 ± 0.001	100	1.01 ± 0.05	100	–	0.44 ± 0.03	100	–	–	–	1.11 ± 0.07	100
Amph. 20 mg/kg × 2 for 18 days (n = 6)	0.07 ± 0.003[a]	40	0.51 ± 0.01[a]	50	–	0.17 ± 0.01[a]	39	–	–	–	0.58 ± 0.02[a]	52

[a] Different from controls: $P < 0.001$.
[b] Different from controls: $P < 0.01$.
[c] Values taken from fig. 5.

Changes in HVA levels of the caudate nucleus.

HVA in the caudate nucleus was 1.53±0.10 μg/g in the control animals. Amphetamine, 20 mg/kg given intraperitoneally, caused a decrease in the HVA levels to about 45 % of the control value at 2–4 hours after the injection (fig. 5). At 24 hours after amphetamine administration there was no statistically significant difference in the HVA levels between amphetamine treated and control guinea pigs.

Tissue levels of amphetamine, CA and HVA after chronic amphetamine treatment.

In table 3 the results of three different experiments are summarized. The first experiment shows the amphetamine concentrations and the changes in CA and HVA levels at 4 hours after 1 injection of amphetamine. In the second and third experiments amphetamine (20 mg/kg given intraperitoneally) or saline was given twice daily for 7 days and 18 days respectively and the animals were sacrificed 4 hours after the last amphetamine injection.

It was found that the decrease in brain and heart NA to 63–65 % after one injection was pronounced (23–48 %) after chronic amphetamine treatment for 7 days. Simultaneously the brain concentration of amphetamine

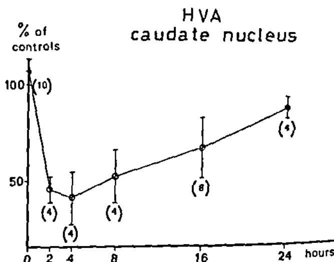


Fig. 5. Effect of dl-amphetamine-SO_4, 20 mg/kg intraperitoneally, on the level of homovallinic acid (HVA) in the caudate nucleus. Values are expressed as percentages (mean ± S. E. M.) of the control level (1.53 ± 0.10 μg/g). Open circles indicate statistically significant differences ($P < 0.05$–0.001) from the control values. Figures in brackets = number of observations

was doubled. There was no further decrease in brain NA after 18 days of chronic amphetamine treatment. Brain DA, which was only slightly reduced after 1 injection decreased to 50 % of the control level after 18 days of chronic amphetamine treatment. Moreover, the HVA level of the caudate nucleus decreased to 22 % of the control value after one week of chronic amphetamine treatment as compared with 42 % after a single injection of the drug.

Discussion

Elimination and metabolism of amphetamine.

The concentrations of amphetamine in the brain and plasma in guinea pigs have a time-course different from that in the rat. According to the present results in guinea pigs, amphetamine is successively absorbed from the peritoneal cavity during the first two hours reaching peak levels at 2 hours after the administration. The disappearance of amphetamine seems to be exponential for the following 14 hours with an apparent half-life of 2.5–3.1 hrs. A similar figure was preliminarily reported by GROPPETTI & COSTA (1969b). In rats maximal values are already found at 15 minutes after the intraperitoneal injection of various doses of amphetamine, and the disappearance curve of the drug has a multi-exponential course with an initial half-life of approximately 40 minutes (MAICKEL *et al.*, SEGAL & RUSSEL 1969; LEWANDER 1971a). The difference in the disappearance rates between the species indicates that the metabolism of amphetamine is more rapid in the rat than in the guinea pig. The main metabolic pathway of amphetamine in rats is parahydroxylation (AXELROD 1954) and in guinea pigs, deamination (DRING *et al.* 1970; present study). Although about equal amounts of an injected dose of 20 mg/kg of amphetamine is excreted (54 %/24 hrs in rats and 43 %/16 hrs in guinea pigs), 75 % of the excreted radioactivity in the rat is metabolites (LEWANDER 1969) while the corresponding figure for the guinea pig is 45 % (table 1). This observation shows that besides differences in the metabolic pathways of amphetamine there is also a difference in the rate of metabolism between the species. Differences in urinary pH between the species (7.8–8.5 in the guinea pig; 5.8–6.8 in rats (LEWANDER 1969)) may also contribute to variations in the renal elimination rate of amphetamine (ASATOOR *et al.* 1965; BECKETT & ROWLAND 1965; BECKETT 1969; ROWLAND 1969).

The brain/plasma concentration ratio was 16 in the guinea pig as compared with 8–9 in the rat (MAICKEL *et al.* 1969, LEWANDER 1971a). The central stimulant effects of amphetamine as measured by motor activity counts and observed stereotype behaviour closely followed the brain and plasma levels of the drug. With the disappearance of the behavioral signs of central excitation (approximately 10 hrs after injection) the brain concentration of amphetamine

was 1–2 μg/g, which therefore seems to be the minimum level needed to elicit these effects of amphetamine in the guinea pig.

After one week of chronic amphetamine administration (12 hour intervals) the brain concentration of amphetamine was increased as compared with the acute experiment (table 3). The accumulation of amphetamine in the tissues is probably due to the rather long $t_{1/2}$ of the drug in this species.

Relationship between amphetamine and catecholamine stores.
Amphetamine caused an increase in the urinary excretion of NA and A in agreement with previous findings in rats (BISCARDI *et al.* 1964; BEAUVALLET *et al.* 1966; LEWANDER 1968a & b). Both brain and heart levels of NA were reduced to the same extent by amphetamine. The reduction appeared to persist as long as amphetamine was present in the tissues. The finding that the brain stem NA and heart NA values had not reached control levels at 24 hrs may be due to the presence of amphetamine in the brain and plasma at that time.

In rats the depletion of brain and heart NA is partly due to the incorporation into the NA storage granules of a metabolite of amphetamine, *p*-hydroxynorephedrine, as a false transmitter (GOLDSTEIN & ANAGNOSTE 1965; THOENEN *et al.* 1966; GROPPETTI & COSTA 1969a; LEWANDER 1970 & 1971a; BRODIE 1970; COSTA & GROPPETTI 1970). However, amphetamine caused NA depletion even in rats pretreated with desmethylimipramine (LEWANDER 1968b & 1971a; GROPPETTI & COSTA 1969a), a drug which inhibits *p*-hydroxylation of amphetamine in rats (CONSOLO *et al.* 1967; LEWANDER 1968b). In guinea pigs amphetamine is not p-hydroxylated (DRING *et al.* 1970; present study) and *p*-hydroxynorephedrine (or norephedrine) could not be detected in the guinea pig brain and heart tissues in agreement with GROPPETTI & COSTA (1969b). Therefore, the amphetamine-induced NA depletion in guinea pigs, as well as in rats pretreated with desmethylimipramine, has to be explained by other mechanisms, e. g. release of granular bound NA (CARLSSON *et al.* 1965; EULER & LISHAJKO 1968) in combination with inhibition of granular uptake or preferential release of newly synthezised NA (EULER & LISHAJKO 1968; BESSON *et al.* 1969; GLOWINSKI 1970).

Brain DA was only slightly decreased in the guinea pig after a single injection of amphetamine. In rats the DA level in the brain is largely unaffected or even increased (MCLEAN & MCCARTNEY 1961; BAIRD & LEWIS 1964; MOORE & LARIVIERE 1963; GUNNE & LEWANDER 1967 & 1968; LEWANDER 1968a & b, 1971a).

An extraneuronal release of brain NA and DA caused by amphetamine is indicated by the finding of an increase in brain NM and MTA levels after amphetamine in nialamide-pretreated guinea pigs. This observation confirms similar results obtained previously in rats (see Introduction). An amphetamine induced release of brain NA and DA has also been recorded in rats by other

more direct methods (McKenzie & Szerb 1968; Stein & Wise 1969; Besson *et al.* 1969; Besson *et al.* 1969; Wise & Stein 1970) and cats (Carr & Moore 1969).

Chronic amphetamine treatment for 1 or 2.5 weeks was followed by a more pronounced depletion of both brain and heart NA as well as brain DA. The enhanced depletion of brain CA after one week of chronic amphetamine might be explained by the high concentrations of amphetamine in the brains of these animals. After 18 days of chronic treatment there was no further depletion of brain or heart CA, which might indicate that the brain amphetamine concentration had reached a steady state level within the first week of repeated amphetamine injections. The results of the experiments on the effect of chronic amphetamine treatment on the brain and heart CA in the guinea pig are in good agreement with previous findings in the rat (McLean & McCartney 1961; Gunne & Lewander 1967; Lewander 1968a).

Effect of amphetamine on the level of HVA in the caudate nucleus.
Since it has been repeatedly demonstrated that amphetamine causes a release of brain DA (cf. above) it was thought of interest to measure the level of brain HVA in similar experiments. According to the present results in guinea pigs amphetamine induced a pronounced decrease in HVA in the caudate nucleus. The time-response curve appeared to follow the time course of the amphetamine concentrations in the brain. After chronic amphetamine treatment for one week there was also a decrease in HVA in the caudate nucleus. Inhibition of MAO caused by amphetamine (Blaschko *et al.* 1937; Glowinski *et al.* 1966) might be the most probable explanation for this finding. Other explanations, e.g. an amphetamine induced increase in the brain to blood transport of HVA, cannot be ruled out at present.

At variance with the present decrease in striatal HVA in the guinea pig is the previously reported increase obtained in cats (Laverty & Sharman 1965) and rats (Jori & Bernardi 1969) after a single injection of amphetamine. However, after repeated injections of amphetamine (Jori & Bernardi 1969) the striatal HVA is also decreased in rats. One explanation for the discrepancy between rats and guinea pigs with regard to the acute effect of amphetamine on brain HVA would be the rapid appearance and rapid disappearance of amphetamine in the rat brain as compared with the conditions in the guinea pig. Studies on the dose-response and time-response relationships between the effects of amphetamine on deamination and extraneuronal release of brain DA might solve this problem.

Main conclusions.
The present experiments in guinea pigs were undertaken in order to study the effects of amphetamine on tissue CA levels in a species which, unlike the rat,

was 1–2 μg/g, which therefore seems to be the minimum level needed to elicit these effects of amphetamine in the guinea pig.

After one week of chronic amphetamine administration (12 hour intervals) the brain concentration of amphetamine was increased as compared with the acute experiment (table 3). The accumulation of amphetamine in the tissues is probably due to the rather long $t_{1/2}$ of the drug in this species.

Relationship between amphetamine and catecholamine stores.
Amphetamine caused an increase in the urinary excretion of NA and A in agreement with previous findings in rats (Biscardi *et al.* 1964; Beauvallet *et al.* 1966; Lewander 1968a & b). Both brain and heart levels of NA were reduced to the same extent by amphetamine. The reduction appeared to persist as long as amphetamine was present in the tissues. The finding that the brain stem NA and heart NA values had not reached control levels at 24 hrs may be due to the presence of amphetamine in the brain and plasma at that time.

In rats the depletion of brain and heart NA is partly due to the incorporation into the NA storage granules of a metabolite of amphetamine, *p*-hydroxynorephedrine, as a false transmitter (Goldstein & Anagnoste 1965; Thoenen *et al.* 1966; Groppetti & Costa 1969a; Lewander 1970 & 1971a; Brodie 1970; Costa & Groppetti 1970). However, amphetamine caused NA depletion even in rats pretreated with desmethylimipramine (Lewander 1968b & 1971a; Groppetti & Costa 1969a), a drug which inhibits *p*-hydroxylation of amphetamine in rats (Consolo *et al.* 1967; Lewander 1968b). In guinea pigs amphetamine is not p-hydroxylated (Dring *et al.* 1970; present study) and *p*-hydroxynorephedrine (or norephedrine) could not be detected in the guinea pig brain and heart tissues in agreement with Groppetti & Costa (1969b). Therefore, the amphetamine-induced NA depletion in guinea pigs, as well as in rats pretreated with desmethylimipramine, has to be explained by other mechanisms, e. g. release of granular bound NA (Carlsson *et al.* 1965; Euler & Lishajko 1968) in combination with inhibition of granular uptake or preferential release of newly synthezised NA (Euler & Lishajko 1968; Besson *et al.* 1969; Glowinski 1970).

Brain DA was only slightly decreased in the guinea pig after a single injection of amphetamine. In rats the DA level in the brain is largely unaffected or even increased (McLean & McCartney 1961; Baird & Lewis 1964; Moore & Lariviere 1963; Gunne & Lewander 1967 & 1968; Lewander 1968a & b, 1971a).

An extraneuronal release of brain NA and DA caused by amphetamine is indicated by the finding of an increase in brain NM and MTA levels after amphetamine in nialamide-pretreated guinea pigs. This observation confirms similar results obtained previously in rats (see Introduction). An amphetamine induced release of brain NA and DA has also been recorded in rats by other

Biscardi, A. M., A. Carpi & O. A. Orsingher: Urinary excretion of catecholamines in the rat after their liberation by reserpine or dexamphetamine. *Brit. J. Pharmacol.* 1964, **23**, 529–539.

Blaschko, H., D. Richter & H. Schlossman: The oxidation of adrenaline and other amines. *Biochem. J.* 1937, **31**, 2187–2196.

Brodie, B. B., A. K. Cho & G. G. Gessa: Possible role of p-hydroxynorephedrine in the depletion of norepinephrine induced by d-amphetamine and in tolerance to this drug. In: *International symposium on amphetamine and related compounds.* Ed.: E. Costa & S. Garattini. Raven Press, New York, 1970, pp. 217–230.

Carlsson, A. & B. Waldeck: A fluorimetric method for the determination of dopamine (3-hydroxytyramine). *Acta physiol. scand.* 1958, **44**, 293–298.

Carlsson, A. & B. Waldeck: A method for the fluorimetric determination of 3-methoxytyramine in tissues and the occurrence of this amine in brain. *Scand. J. Clin. Lab. Invest.* 1964, **16**, 133–138.

Carlsson, A., M. Lindqvist, A. Dahlström, K. Fuxe & D. Masuoka: Effects of the amphetamine group on intraneuronal brain amines *in vivo* and *in vitro*. *J. Pharm. Pharmacol.* 1965, **17**, 521–523.

Carlsson, A., K. Fuxe, B. Hamberger & M. Lindqvist: Biochemical and histochemical studies on the effects of imipramine-like drugs and (+)-amphetamine on central and peripheral catecholamine neurons. *Acta physiol. scand.* 1966, **67**, 481–497.

Carr, L. A. & K. E. Moore: Norepinephrine release from brain by d-amphetamine *in vivo*. *Science* 1969, **164**, 322–323.

Chang, C. C.: A sensitive method for spectrophotofluorimetric assay of catecholamines. *Int. J. Neuropharmacol.* 1964, **3**, 643–649.

Consolo, S., E. Dolfini, S. Garattini & L. Valzelli: Desipramine and amphetamine metabolism. *J. Pharm. Pharmacol.* 1967, **19**, 253–256.

Costa, E. & A. Groppetti: Biosynthesis and storage of catecholamines in tissues of rats injected with various doses of *d*-amphetamine. In: *International symposium on amphetamines and related compounds.* Ed.: E. Costa & S. Garattini, Raven Press, New York, 1970, pp 231–256.

Dingell, J. V., M. L. Owens, M. R. Norvich & F. Sulser: On the role of norepinephrine biosynthesis in the central action of amphetamine. *Life Sci.* 1967, **6**, 1155–1162.

Dring, L. G., R. L. Smith & R. T. Williams: The metabolic fate of amphetamine in man and other species. *Biochem. J.* 1970, **116**, 425–435.

Ellison, T., L. Gutzait & E. J. van Loon: The comparative metabolism of d-amphetamine-^{14}C in the rat, dog and monkey. *J. Pharmacol. exp. Ther.* 1966, **152**, 383–387.

Euler, U. S. v. & F. Lishajko: Improved technique for the fluorimetric estimation of catecholamines. *Acta physiol. scand.* 1961, **51**, 348–355.

Euler, U. S. v. & F. Lishajko: Effect of directly and indirectly acting sympathomimetic amines on adrenergic transmitter granules. *Acta physiol. scand.* 1968, **73**, 78–92.

Glowinski, J. & J. Axelrod: Effects of drugs on the uptake, release and metabolism of H^3-norepinephrine in the rat brain. *J. Pharmacol. exp. Ther.* 1965, **149**, 43–49.

Glowinski, J., J. Axelrod & L. L. Iversen: Regional studies of catecholamines in the rat brain IV. Effects of drugs on the disposition and metabolism of H^3-norepinephrine and H^3-dopamine. *J. Pharmacol. exp. Ther.* 1966, **153**, 30–41.

Glowinski, J.: Effects of amphetamine on various aspects of catecholamine metabolism in the central nervous system of the rat. In: *International symposium on amphetamines and related compounds.* Ed: E. Costa & S. Garattini, Raven Press, New York, 1970, pp. 301–316.

Goldstein, M. & B. Anagnoste: The conversion *in vivo* of D-amphetamine to (+)-*p*-hydroxynorephedrine. *Biochem. Biophys. Acta* 1965, **107**, 166–168.

Groppetti, A. & E. Costa: Tissue concentration of *p*-hydroxynorephedrine in rats injected with d-amphetamine: Effect of pretreatment with desipramine. *Life Sci.* 1969a, **8**, 653–665.

Groppetti, A. & E. Costa: *d*-Amphetamine (A): Metabolites and depletion of brain and heart norepinephrine (NE) in guinea pig and rat. *Fed. Proc.* 1969b, **28**, 795.

Gunne, L.-M. & T. Lewander: Long-term effects of some dependence-producing drugs on the brain monoamines: In: *Molecular basis of some aspects of mental activity.* Academic Press, London and New York, 1967, **2**, 75–81.

Gunne, L.-M. & T. Lewander: Brain catecholamines during chronic amphetamine intoxication. In: *The addictive states.* A.R.N.M.D. The Williams & Wilkins Company, Baltimore, 1968, vol. XLVI, pp. 106–112.

Hansson, L. C. F.: Evidence that the central action of (+)-amphetamine is mediated via catecholamines. *Psychopharmacologia* (Berl.) 1966, **10**, 289–297.

Häggendal, J.: Fluorimetric determination of 3-O-methylated derivatives of adrenaline and noradrenaline in tissues and body fluids. *Acta physiol. scand.* 1962, **56**, 258–266.

Jori, A. & D. Bernardi: Effect of amphetamine and amphetamine-like drugs on homovanillic acid concentration in the brain. *J. Pharm. Pharmacol.* 1969, **21**, 694–697.

Korf, J., B.-E. Roos & B. Werdinius: An improved fluorimetric method for determination of homovanillic acid in tissues using column chromatographic separation *Scand. J. Clin. Lab. Invest.* 1970. In press.

Laverty, R. & D. F. Sharman: Modification by drugs of the metabolism of 3,4-dihydroxyphenylethylamine, noradrenaline and 5-hydroxytryptamine in the brain. *Brit. J. Pharmacol.* 1965, **24**, 759–772.

Lewander, T.: Urinary excretion and tissue levels of catecholamines during chronic amphetamine intoxication. *Psychopharmacologia* 1968a, **13**, 394–407.

Lewander, T.: Effects of amphetamine on urinary and tissue catecholamines in rats after inhibition of its metabolism with desmethylimipramine. *Europ. J. Pharmacol.* 1968b, **5**, 1–9.

Lewander, T.: Influence of various psychoactive drugs on the *in vivo* metabolism of d-amphetamine in the rat. *Europ. J. Pharmacol.* 1969, **6**, 38–44.

Lewander, T.: Catecholamine turn-over studies in chronic amphetamine intoxication. In: *International symposium on amphetamines and related compounds.* Ed.: E. Costa & S. Garattini, Raven Press, New York, 1970, pp. 317–330.

Lewander, T.: On the presence of *p*-hydroxynorephedrine in the rat brain and heart in relation to changes in catecholamine levels after administration of amphetamine *Acta pharmacol. et toxicol.* 1971a, **29**, 33–48.

Lewander, T.: Displacement of brain and heart noradrenaline by *p*-hydroxynorephedrine after administration of *p*-hydroxyamphetamine. *Acta pharmacol et toxicol* 1971b, **29**, 20–32.

Maickel, R. P., R. H. Cox Jr., F. P. Miller, D. S. Segal & R. W. Russel: Correlation of brain levels of drugs with behavioural effects. *J. Pharmacol. exp. Ther.* 1969, **165**, 216–224.

McKenzie, G. M. & J. C. Szerb: The effect of dihydroxyphenylalanine, pheniprazine and dextroamphetamine on the *in vivo* release of dopamine from the caudate nucleus. *J. Pharmacol. exp. Ther.* 1968, **162**, 302–308.

McLean, J. R. and M. McCartney: Effect of d-amphetamine on rat brain noradrenaline and serotonin. *Proc. Soc. exp. Biol.* (N. Y.). 1961, **107**, 77–79.

Moore, K. E., & E. W. Larivière: Effects of d-amphetamine and restraint on the content of norepinephrine and dopamine in rat brain. *Biochem. Pharmacol.* 1963, **12**, 1283–1288.

Quinton, R. M. & G. Halliwell: Effects of β-methylDOPA and DOPA on the amphetamine excitatory response in reserpinized rats. *Nature* (Lond.) 1963, **200**, 178–179.

Randrup, A & I. Munkvad: Role of catecholamines in the amphetamine excitatory response. *Nature* (Lond.) 1966, **211**, 5048.

Rowland, M.: Amphetamine blood and urine levels in man. *J. Pharm. Sci.* 1969, **58**, 508–509.

Scheel-Krüger, J. & A. Randrup: Stereotype hyperactive behaviour produced by dopamine in the absence of noradrenaline. *Life Sci.* 1967, **6**, 1389–1398.

Stein, L. & C. D. Wise: Release of norepinephrine from hypothalamus and amygdala by rewarding medial forebrain bundle stimulation and amphetamine. *J. comp. physiol. Psychol.* 1969, **67**, 189–198.

Thoenen, H., A. Hürliman, K. F. Gey & W. Haefely: Liberation of *p*-hydroxynorephedrine from cat spleen by sympathetic nerve stimulation after pretreatment with amphetamine. *Life Sci.*, 1966, **5**, 1715–1722.

Weissman, A. & B. K. Koe: Behavioural effects of l-α-methyltyrosine, and inhibitor of tyrosine hydroxylase. *Life Sci.* 1965, **4**, 1037–1048.

Wise, C. D. & L. Stein: Amphetamine: facilitation of behaviour by augmented release of norepinephrine from the medial forebrain bundle. In: *International symposium on amphetamines and related compounds.* Ed.: E. Costa & S. Garattini, Raven Press, New York, 1970, pp 463–486.

Acta pharmacol. et toxicol. 1971, 29, 226–240.

From the Department of Pharmacology, Queen's University,
Kingston, Ontario, Canada

The 100-Day LD50 Index of Captan

By

Eldon M. Boyd and Eva Carsky

(*Received July 7, 1970*)

Abstract: Captan was given by gavage in a range of doses once daily for 100 days to young male albino rats. The oral LD50 (100 days) ± S. E. M. or daily dose which killed 50 % of rats over this period was found to be 0.92 ± 0.23 g/kg/day. The oral 100-day LD50 index, or oral LD50 (100 days) expressed as a percentage of the acute oral LD50 (1 dose) was 7.3 ± 1.9 which indicated that on chronic administration captan was the most toxic of 12 drugs for which published values of the 100-day LD50 index are available. Signs of toxicity were most evident during the first 3 weeks of daily administration and were due to a severe gastroenteritis, an inflammatory reaction in many organs, and degenerative changes in other organs.

Key-words: Captan, chronic oral toxicity – fungicides, captan – pesticides, captan – toxocity, chronic, 100-day LD50 index.

The research to be described in this communication is the second in a series of studies on the multiposal ("subacute," "chronic") toxicity on pesticides. The first study was on the multiposal toxicity of dicophane (chlorphenotanum NFN) or DDT by Boyd & Lin (1970). In both of these studies, the animals used (albino rats) were fed a standard laboratory chow. In subsequent studies, the multiposal toxicity of dicophane and captan will be determined in rats fed from weaning on protein-deficient diets. The programme was undertaken at the request of the World Health Organization in order to obtain data relative to the safety of pesticides for use in underdeveloped countries of the world where the diet is usually low in protein, particularly animal protein. In preliminary studies, the uniposal or acute oral toxicity of a series of pesticides was determined in albino rats fed from weaning on diets deficient in protein. It was found that the animals became increasingly more susceptible to the acute oral toxic effects of pesticides as the dietary protein was progressively lowered (Boyd 1969). The increase in acute toxicity was minor for dicophane and marked for captan (Boyd *et al.* 1970). Hence dicophane and captan were selected for studies of multiposal toxicity.

Captan is an N-trichloromethylthio derivative of partially saturated phthalidimide used mainly as a protectant fungicide (SPENCER 1968) which appears to act by combination with cysteine and glutathione groups in proteins (MENZIE 1969). BOYD & KRIJNEN (1968) reported the acute oral LD50 of captan in young, male albino rats fed laboratory chow to be 12.5 ± 3.5 g per kg body weight. In the acute studies, death mostly occurred during the third and fourth days, with the animals in hypothermic coma, and was associated with congestion and dehydration of many organs, infiltrative ulcers in the cardiac part of the stomach, a stress reaction and degenerative changes in the kidneys and other organs.

Methods

The experiments were performed on 116 young, male albino rats of a Wistar strain, free of endemic pneumonia, purchased from Woodlyn Farms, Limited, of Guelph, Ontario with an initial body weight of 220 ± 18 g (mean ± S. D.). They were fed Rockland Rat Diet Complete obtained from Teklad, Incorporated, of Winfield, Iowa. The composition of the diet has been described by BOYD & LIN (1970).

Captan was provided by the Chevron Chemical Company of San Francisco, California, as a technical grade which assayed 92 % pure. Being insoluble in water, it was dissolved in cottonseed oil and the solution administered intragastrically through a cannula attached to a syringe in a constant volume of 20 ml/kg body weight. This volume of cottonseed oil had been reported by BOYD & BOULANGER (1969) to be nontoxic to albino rats and the volume was kept constant for reasons given by BOYD (1968).

Doses of captan for daily oral administration were selected from the oral LD50 (1 dose) according to the methods reviewed by BOYD (1971). Each dose was given to 20 animals for 100 days or until all the animals were dead The controls received 20 ml of cottonseed oil per kg body weight per day. Except as noted below, the total weekly dose was divided into 5 equal parts, each of which was given daily for 5 days, i. e. Monday through Friday, with no treatment on Saturday and Sunday. When the duration of treatment exceeded one week, the daily dose given 5 days a week was multiplied by 5/7 to obtain the average daily dose.

In a separate experiment, a weekly dose of 15 g/kg/week was divided into 5 equal parts and given as 3 g/kg/day for 5 days a week and the results compared with those of the same total weekly dose given as 2.14 g/kg/day each day for 7 days a week. Treatment was given once each day except in another separate experiment in which a daily dose of 6 g/kg/day was given as 3 g/kg in the morning and 3 g/kg in the late afternoon and the results compared with those obtained when the total daily dose of 6 g/kg was given once (in the morning) a day.

The animals were housed in boarding cages, 6 to 8 rats per cage, with food and water ad libitum, during daily dosing with captan. The experiments were performed during the autumn and winter months of the year.

At weekly intervals, each animal was placed in a metabolism cage, one rat per cage, for recording clinical measurements over a period of 24 hours. Clinical measurements included body weight gain (or loss) per week in g, food consumption in g food per kg body weight per 24 hours, water intake in ml/kg/24 hours, colonic temperature in °F, urinary volume in ml/kg/24 hours, urinary blood in units/kg/24 hours, urinary glucose

and protein output in mg/kg/24 hours and urinary pH on 24-hour samples. Clinical signs of intoxication were recorded in clinical units of 1+ to 4+.

An autopsy was performed on all the rats which died and the gross pathology recorded. Histopathology was recorded on any organs which appeared abnormal on gross examination and the organs listed in table 2 below of representative animals from each dose group. Microscopic examination was done on sections stained with haematoxylin-phloxine-saffron from the blocks of tissue fixed in Lillie's buffered formalin. The fresh wet weight and water content of organs listed in table 3 below were recorded in all the animals which survived to 100 days and in the controls at 100 days.

Weight was recorded in g. The contents of the lumen of the gastrointestinal tract were removed by a standardized process of waterwashing, milking, and filter paper drying before recording weight. The sample of skeletal muscle was the right half of the muscular layer of the ventral abdominal wall. The sample designated 'skin' included the tail which did not homogenize well with the residual carcass. The weight was measured on a Mettler semimicrogramatic balance to 0.1 mg on all organs except the skin and residual carcass which were weighed to 0.01 g.

The water content was determined on weighed aliquots of the organs dried to constant weight in a Fisher forced-draft, temperature-controlled oven at 90°. The sample of skin for water analysis was taken from the dorsolumbar region after clipping off the hair. The residual carcass, after removing the organs listed in table 3 below, was cut into small pieces, homogenized in a Waring blendor, and an aliquot weighed for water analysis. The results were calculated as g water per 100 g dry weight of tissue.

The results were analyzed statistically by the application of t-tests to differences between means and to regression analysis on dose, effect or interval of administration The LD50 (100 days) ± S. E. M. was calculated as described by BOYD (1971) and BOYD & LIN (1970). Details of technique have been reviewed by BOYD (1968).

Results

The LD50 (100 days) ± S. E. M., or daily dose which killed 50% of animals when given over a period of 100 days, was found to be 0.916 ± 0.233 g/kg/day. It was calculated from the equation $Y = (3.526 - 1.305 \log X) \pm 0.233$ where Y is the LD50 calculated at days indicated on X; at 100 days, for example, log X is 2. In a similar manner, the maximal LD0 (100 days), which is equivalent to about the LD1 (100 days) in this study, was estimated at 0.461 ± 0.162 g/kg/day and the minimal LD100 (100 days), or LD99 (100 days) to be 2.208 ± 0.567 g/kg/day.

The clinical signs of toxicity are summarized in table 1 as average changes over intervals of 3 weeks. The toxicity syndrome was most marked during the first 3 weeks of captan administration. During the initial 3 weeks, all the changes shown in table 1 as being statistically significant were greater the greater the daily dose of captan with the exception of water intake. During the first week, for example, loss of body weight in rats given the largest dose of captan was 7 times the gain in weight of the controls while at the lowest dose of captan the loss was twice the gain in the controls. Most deaths also occurred during the first 3 weeks.

The toxicity syndrome subsided at 22 to 42 days in survivors which, at this time, exhibited only a mild hypothermia and aciduria.

From day 43 to 100, captan-treated survivors actually ate more food than the controls. Pair-fed controls were not introduced, however, because the average food intake of captan-treated rats over the whole period of 100 days was not significantly different from that in the controls given cottonseed oil only. *In spite of the augmented food intake, the growth of captan-treated* rats was not greater at 43 to 100 days than the growth of controls, and at 100 days captan-treated survivors weighed some 30 % less than the controls. In this final period of 57 days, as noted in table 1, the captan-treated survivors suffered from diuresis, polydipsia, and a slight fever with a return of urinary pH toward, but not to, normal. At no time were there any significant changes in urinary blood, glucose or protein.

Table 1.

Clinical observations in albino rats given captan daily by gavage.

Observation	Days 1 to 21	Days 22 to 42	Days 43 to 63	Days 64 to 84	Days 85 to 100
Gain in body weight:					
g[a]	− 285**	+ 11	+ 24	− 14	− 10
Food intake:					
g/kg/24 hours[a] . ..	− 29*	0	+ 2	+ 44**	+ 22*
Water intake:					
ml/kg/24 hours[a]...	+ 47*	+ 29	+ 178*	+ 464**	+ 403**
Colonic temperature·					
°F[a]	− 2.0**	− 1.0*	− 0.1	*1.4*	+ 1.9**
Urinary volume:					
ml/kg/24 hours[a]...	+ 39	+ 21	+ 130**	*158**	+ 124**
Urinary pH:					
24 hour samples[a]	− 15**	− 19**	− 19**	− 13**	− 10**
Soiled skin[b]	+ 1.2**	0 0	0 0	0 0	0 0
Prostration[b]	+ 1.1**	0 0	0.0	0.0	0.0
Loose stools[b]	+ 0 5*	0.0	0 0	0.0	0.0
Haemorhinorrhea[b] ..	+ 0.3	0.0	0.0	0.0	0 0

[a]) The results are expressed as the mean percent change from controls, specifically as $((\bar{X}_d - \bar{X}_c)/\bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in drug (captan)-treated rats and $\bar{X}_c$ in controls given only cottonseed oil.

[b]) Measured in clinical units of 1 + to 4 + and expressed as mean difference from controls, the sign usually being absent in controls.

* Significantly different from results in controls at P = 0.05 to 0 02.

** Significantly different from results in controls at P = 0.01 or less.

Table 2.

Histopathological observations at death due to daily oral administration of captan to albino rats and in survivors at 100 days.

Organ	At death during first 21 days	In survivors at 100 days
Adrenal glands	*Lipoid droplets prominent in cortex*	*Lipoid droplets prominent*
Brain	*Areas of capillary-venous congestion of cerebrum, cerebellum and meninges*	*Mild hyperaemia*
Gastrointestinal tract:		
Cardiac stomach	*Congestion of the lamina propria with infiltrative ulcers*	*Hypertrophy of the stratified squamous epithelium*
Pyloric stomach	Congestion with haemorrhagic necrotic ulcers	Normal appearance
Small bowel	Congestion of the villi and submucosa	Hypertrophy and hyperaemia
Caecum	Capillary-venous congestion of the lamina propria and submucosa	Hypertrophy and hyperaemia
Colon	*Mild congestion*	Normal appearance
Heart	*Normal appearance*	*Normal appearance*
Kidneys	*Capillary-venous congestion in the region of the loop of Henle, occasional tubular cloudy swelling and infection*	*Mild cloudy swelling of the tubules*
Liver	Diffuse cloudy swelling	Normal appearance
Lungs	Oedema, congestion, venous thrombosis and occasionally pneumonitis	Areas of capillary-venous congestion
Muscle (ventral abdominal wall)	Normal appearance	Normal appearance
Salivary (submaxillary) glands	Deficiency of serous *zymogenic granules*	*Minor deficiency of zymogenic granules*
Skin	*Normal appearance*	Normal appearance
Spleen	Red pulp atrophied, *trabeculae prominent*	Normal appearance
Testes	Marked inhibition of *spermatogenesis, interstitial congestion*	Deficiency of normal sperm
Thymus gland	Marked loss of thymocytes, atrophy	Marked loss of *thymocytes, atrophy*

Pathological observations observed at autopsy in animals which died during the first 3 weeks are summarized in table 2. Captan produced a marked local irritant inflammatory reaction in the gastrointestinal tract. The stratified squamous epithelium of the cardiac stomach was pierced by ulcers infiltrated heavily by granulocytes as illustrated in fig. 1. Ulcers in the glandular or pyloric stomach took the form of bleeding, necrotic areas in the gastric glands as illustrated in fig. 2. There was capillary-venous congestion in the lamina propria and submucosa of the caecum and colon. The colon is normally resistant to a local inflammatory reaction to drugs given chronically by gavage and the changes observed in this study are shown in fig. 3.

The presumed presence of large amounts of captan in the blood following absorption, produced an inflammatory reaction in the form of capillary-venous congestion in many organs such as the cerebrum, cerebellum, meninges, kidneys, lungs and testes. In the lungs, congestion was accompanied by venous thrombosis, perivascular oedema and occasionally pneumonitis.



Fig. 1. A photomicrograph of the cardiac stomach of an albino rat which died on the 8th day of daily oral administration of captan showing an early infiltrative ulcer penetrating the stratified squamous epithelium and coagulating the thick layer of covering mucus. On the abluminal side there is haemorrhage and necrosis of the muscularis mucosae. (Stain: haematoxylin-phloxine-saffron; magnification × 100).

Along with the general inflammatory reaction there were degenerative changes in certain organs such as the kidneys, liver, salivary glands and testes. Inhibition of spermatogenesis was particularly marked as illustrated in fig. 4. There was a stress reaction in the adrenal and thymus glands and spleen. The spleen consisted mostly of lymphocytic white pulp with prominent trabeculae, the red pulp being markedly deficient. The skeletal muscle fibres were small with an excess of connective tissue separating the myofibrils as illustrated in fig. 5.



Fig. 2. A photomicrograph of the pyloric stomach of a rat which died on the 7th day of daily oral administration of captan showing a small necrotic ulcer in the luminal half of the gastric glands with some haemorrhage at its base (*Stain*: haematoxylin-phloxine-saffron; *magnification* × 100).



Fig 3. A photomicrograph of the colon of a rat which died on the 13th day of daily oral administration of captan showing vascular congestion of the submucosa between the muscularis mucosae and muscularis externa (Stain: haematoxylin-phloxine-saffron; magnification × 100).

The pathological reaction was less marked in rats which survived for 100 days as indicated in table 2. The mucosa of the gastrointestinal tract had reacted to the presence of irritant amounts of captan by an adaptive hypertrophy. End organs of toxicity such as the brain, kidneys, lungs and testes had adapted to the presence of large amounts of captan in the blood and there was much less inflammation. The degenerative changes seen in animals which died during the first three weeks were less evident in the survivors at 100 days. The stress reaction was less marked except in the thymus gland which remained atrophied.

Changes in the wet weight and water content of body organs in survivors at 100 days are summarized in table 3. Hypertrophy of parts of the gastrointestinal tract was reflected in an increase in wet weight with variable changes in water levels. Loss of body weight was seen to be due mainly to loss of wet weight in muscle and skin. There was a significant increase in the water content of the skin, indicating a still greater loss of dry weight. Loss of weight was relatively greatest in the thymus gland. Many organs such as the

adrenal glands, cardiac stomach, liver, lungs, salivary glands and residual carcass, had increased water levels.

Data on treatment for 7 days a week as against 5 days a week are summarized in table 4. It should be noted that at 14 days the various parameters in rats given a weekly dose of 15 g/kg in equal portions each day for 7 days a week were of the same nature as those seen in rats given the same weekly dose in equal portions for 5 days a week. A rather large weekly dose was



Fig. 4. A photomicrograph of the testes of a rat which died on the 18th day of daily oral administration of captan showing a deficiency of spermatogonia, pyknosis of the spermatogenic cells, ghost spermatocytes (no or poorly developed nucleus), no spermatids, no spermatozoa and some congestion of the interstitial tissue (Stain: haematoxylin-phloxine-saffron; magnification × 450).



Fig. 5. A photomicrograph of skeletal muscle from the ventral abdominal wall of a rat which died on the 9th day of daily administration of captan showing excess connective tissue between the myofibrils (Stain: haematoxylin-phloxine-saffron; magnification × 450.)

selected for this comparison since it was considered that differences due to treatment for 7 as against 5 days a week would be more likely to appear from a high dose with a high mortality rate than from a low dose with a low mortality rate. This reasoning could be wrong, however, and it might be desirable to repeat the comparison at lower weekly doses. When the results of this comparison became available, daily doses given 5 days a week were multiplied by 5/7 to obtain average daily doses in studies that extended beyond one week.

In the second separate experiment, it was anticipated that the toxicity of captan would be less given daily as 3 g/kg in the morning and 3 g/kg in the afternoon than when given as 6 g/kg in a single dose in the morning. The results, summarized in table 5, indicated that this was not so. When administered daily as 3 g/kg in the early morning and 3 g/kg in the late afternoon, captan was, if anything, slightly more toxic than when given as 6 g/kg once a day in the morning. This may have been associated with the eating habits of the rats, the animals consuming most of their food at night. The dose

Table 3.

Changes in weight and water content of body organs in albino rats which survived 100 days of oral administration of captan.[a]

Organ	Wet weight	Water content
Adrenal glands	0.0	+ 6.2*
Brain	− 1.2	+ 0.2
Gastrointestinal tract:		
Cardiac stomach	+ 45.2**	+ 5.6*
Pyloric stomach	+ 3.2	+ 0.2
Small bowel	+ 25.3**	− 6.9
Caecum	+ 32.3**	− 12.3**
Colon	+ 3.7	+ 1.2
Heart	− 7.8	+ 1.6
Kidneys	− 1.2	+ 0.6
Liver	+ 2.2	+ 12.3**
Lungs	+ 3.1	+ 7.2**
Muscle (ventral abdominal wall)	− 52.8**	− 0.6
Salivary (submaxillary) glands	+ 7.8	+ 7.8*
Skin	− 46.5**	+ 22.7**
Spleen	− 5.0	+ 2.2
Testes	− 8.9	− 5.8
Thymus gland	− 51.2**	− 2.1
Residual carcass	− 37.6**	+ 7.2*
Autopsy body weight	− 28.7**	

a) Weight was measured in grams and water content in grams water per 100 grams dry weight of tissue. The results are expressed as mean percent change from controls given cottonseed oil but no captan, specifically as $((\bar{X}_d - \bar{X}_c)/\bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in drug (captan)-treated animals as $\bar{X}_c$ in controls.
Significantly different from controls at P = 0.05 to 0.02.
Significantly different from controls at P = 0.01 or less.

given late in the afternoon may have been less diluted by the food in the stomach and therefore was probably absorbed more quickly and thus was relatively more toxic. The other signs of toxicity were identical in both groups.

Discussion

The 100-day LD50 index has been proposed by BOYD (1971) as a single figure estimate of chronic lethal toxicity. The 100-day LD50 index is the LD50 (100 days) expressed as a percentage of the acute LD50 (1 dose). The

Table 4.

Observations at 14 days in albino rats given the same total weekly dose of captan (15 g/kg/week) in equal parts, (a) 7 days a week or (b) 5 days a week. There were 20 animals in each of groups (a) and (b). The results are expressed as mean ± S. D. except that mortality is expressed as a percentage incidence.

Observation	(a) 7 day a week	(b) 5 days a week	Controls (no captan)
Percent mortality	90	90	0
Body weight: g	194 ± 10	215 ± 11	260 ± 14
Food intake: g/kg/24 hours	19 ± 8	12 ± 7	44 ± 8
Water intake: ml/kg/24 hours	142 ± 23	92 ± 15	65 ± 11
Colonic temperature: °F	95.8 ± 0.3	95.0 ± 0.1	97.0 ± 0.2
Urinary volume: ml/kg/24 hours	20 ± 7	18 ± 6	24 ± 7
Urinary protein output: mg/kg/24 hours	4.7 ± 2.8	3.4 ± 1.9	3.3 ± 1.4
Urinary pH: 24 hour sample	6.0 ± 0.7	6.4 ± 0 8	7.8 ± 0.7

Table 5.

Death rates in albino rats given captan by gavage in a daily dose of 6 g/kg as (a) one administration per day and as (b) 3 g/kg in the early morning and 3 g/kg in the late afternoon.

	(a) As one dose per day	(b) As two doses per day
Percent mortality at 2 days	10	25*
Percent mortality at 4 days	70	75
Percent mortality at 6 days	90	95
Hours to death of all animals: mean ± S. D.	119 ± 71	77 ± 28*

* Significantly different from results in group (a) at P = 0 05.

acute oral LD50 (1 dose) of captan in rats fed a diet similar to those employed in the present study was reported to be 12.5±3.5 g/kg by BOYD & KRIJNEN (1968) and the oral LD50 (100 days) was found to be 0.916±0.233 g/kg/day in the studies described here. The oral 100-day LD50 index ot captan may therefore be calculated to be 7.3±1.9 (±S. E. M.). BOYD & LIN (1970) have assembled from the literature values for the 100-day LD50 index on eleven studies performed with ten drugs. The first entry in their table 5, a value of 12.3±4.3 for phenacetin given orally to guinea pigs, was based on a preliminary estimate of BOYD & CARRO-CIAMPI (1970) who later reported the correct value to be 8.6±3.1. This is the lowest of all the indices in table 5 of BOYD & LIN (1970) and the value of 7.3±1.9 found here for captan is even lower. The lower the value of the 100-day LD50 index the smaller the fraction of the acute LD50 (1 dose) which can be given daily for 100 days without producing deaths and therefore the relatively more toxic is the drug or the chemical agent used for chronic daily administration. By this criterion, captan is the most toxic agent for chronic use of all the substances on which a 100 day LD50 index has been calculated to date.

BOYD (1971) has noted that if the chronic LD50 is appreciably decreased at 100 days, continued administration will yield a lower chronic LD50. From the equation used to calculate the oral LD50 (100 days) for captan, it may be estimated that the LD50 (200 days) would be 0.523 g/kg/day and the LD50 (365 days) 0.183 g/kg/day. This would yield a 365-day LD50 index of a little over one, indicating an even more marked toxicity. There are, however, no published values of the 365-day LD50 index for comparison and in any event it is an estimate calculated by extrapolation and therefore may not be correct. In evidence reviewed by FAO/WHO (1965) there was some indication of an increased toxicity of captan with long continued use which took the form of a reduction in body weight of albino rats during the final 4 months of a two year feeding study at 50 mg/kg/day.

These various considerations indicate that while captan has a high acute oral LD50, suggesting safety, under conditions of chronic use, the safety factor markedly decreases. One use of captan is as a soil fungicide and a point in its favour, safety-wise, is that its half-life free in the soil is only 1 to 2 days (GRIFFITH & MATHEWS 1969). However, it resists degradation if it becomes incorporated into seeds implanted in the soil. Captan is related structurally to thalidomide and VERRETT et al. (1969) have shown that concentrations of 3 to 20 p.p.m. in chicken egg yolk produce a high incidence of abnormal embryos.

The key to the understanding of the marked chronic toxicity of captan may lie in its ability to combine with proteins in the end organs where toxicity occurs. MENZIE (1969) has reviewed evidence indicating that the trichloromethylthio group of captan is readily hydrolyzed off the molecule and con-

verted into highly reactive groups such as thiophosgene (CS Cl_2) which combines with and alters amino acids such as cysteine and glutathione. This in turn suggests the hypothesis that toxicity could result when a certain amount or percentage of protein in an organ was so altered. If so, one would anticipate that organs with a rapid turnover of proteins would be very susceptible to captan toxicity and this was found to be true to the extent that there was inhibition of spermatogenesis in the testes. If the hypothesis is correct, removal of protein from the diet would be expected to markedly augment the susceptibility to captan toxicity and KRIJNEN & BOYD (1970) have shown that rats fed no dietary protein from weaning, become an astounding two thousand fold more susceptible to the acute toxic effects of captan.

These various considerations suggest that the present accepted tolerance limits for captan in foodstuffs may be too high. In Canada, CHAPMAN (1967) places the tolerance at 40 p.p.m. From the acute data of KRIJNEN & BOYD (1970) it may be calculated that the tolerance limit should be about 0.1 p.p.m. or 0.01 mg/kg/day. The World Health Organization estimates the acceptable daily intake for man at up to 0.1 mg/kg/day (FAO/WHO 1965). If the results of the present study apply to rats fed from weaning on protein-deficient diets, it can be calculated that the tolerance limit should be lowered to about 0.01 p.p.m. particularly in underdeveloped countries where the diet is usually low in protein.

Acknowledgement

This project was assisted financially by a grant from the World Health Organization.

REFERENCES

Boyd, E. M.: Predictive drug toxicity: Assessment of drug safety before human use. *Canad. med. Ass. J.* 1968, 98, 278–293.

Boyd, E. M.: Dietary protein and pesticide toxicity in male weanling rats. *Bull. Wld. Hlth. Org.* 1969, 40, 801–805.

Boyd, E. M.: 100-Day LD50 index of chronic toxicity *Clin. Toxicol.* 1971, in press.

Boyd, E. M. & M. A. Boulanger: Acute oral toxicity of cottonseed oil. *Toxicol. Appl. Pharmacol.* 1969, 14, 432–438.

Boyd, E. M. & G. Carro-Ciampi. The oral 100-Day LD50 index of phenacetin in guinea pigs. *Toxicol. Appl Pharmacol* 1970, 16, 232–238.

Boyd, E. M. & C. J. Krijnen· Toxicity of captan and protein-deficient diet. *J. clin. Pharmacol.* 1968, 8, 225–234.

Boyd. E. M., C. J. Krijnen & T. K. Tanikella: The influence of a wide range of dietary protein concentration on the acute oral toxicity of malathion. *Arch. Toxikol.* 1970, 26, 125–132.

Boyd, E. M. & F. M. Lin: Maximal tolerated daily doses of dicophane. *Acta pharmacol et toxicol.* 1970, **28**, 149–160.

Chapman, R. A.: *Tolerances for residues of pesticide chemicals.* T. I. L. No. 290, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada, 1967, p. 16.

FAO/WHO: *Evaluation of the toxicity of pesticide residues in food.* FAO Meeting Report No. PL/1965/10/1 and WHO/Food Add./27.65, Food and Agriculture Organization and World Health Organization, United Nations, Geneva, 1965, pp. 28–30.

Griffith, R. L. & S. Mathews: The persistence in soil of the fungicidal seed dressings, captan and thiram. *Ann. Appl. Biol.* 1969, **64**, 113–118.

Krijnen, C. J. & E. M. Boyd: Susceptibility to captan pesticide of albino rats fed from weaning on diets containing various levels of protein. *Fd. Cosmet. Toxicol.* 1970, **8**, 35–42.

Menzie, C. M.: *Metabolism of pesticides.* Special Scientific Report – Wildlife No. 127, Fish and Wildlife Service, Bureau of Sport Fisheries and Wildlife, U. S. Department of the Interior, Washington, D. C., 1969.

Spencer, E. Y.: *Guide to the chemicals used in crop protection.* 5th. edition, Publication 1093, Canada Department of Agriculture, The Queen's Printer, Ottawa, Canada, 1968.

Verrett, M. J., M. K. Mutchler, W. F. Scott, E. F. Reynaldo & J. McLaughlin: Teratogenic effects of captan and related compounds in the developing chicken embryo. *Ann. N. Y. Acad. Sci.* 1969, **160**, 334–343.

Acta pharmacol. et toxicol. 1971, 29, 241–249.

From the Laboratory of Teratology and the Department of Paediatrics, Karolinska institutet, Stockholm, Sweden

The Effect of Salicylate on the Glycogen Content of the Foetal Liver and Heart in Two Strains of Mice

By

Margareta Eriksson

(Received September 25, 1970)

Abstract: Sodium salicylate was injected into A/Jax and CBA mice on the 17th day of gestation in order to study its effect on liver and heart glycogen and its possible correlation with salicylate-induced foetal damage. Glycogen concentration was determined 4, 8 and 24 hours after injection and compared with the values for control animals of the same age. Foetal liver glycogen was reduced for up to 8 hours in A/Jax and CBA mice following an injection of 10 mg/20 g sodium salicylate. This reduction was greater in foetuses with superficial or liver haemorrhages than in foetuses with no haemorrhages. The heart glycogen was also reduced in the injured foetuses. A decrease in foetal glycogen was observed 4 hours after treatment of A/Jax mice with 3 mg/20 g of sodium salicylate. However, at 8 hours the glycogen content had returned to normal levels. The maternal liver glycogen was decreased 4 hours after treatment and then returned to values comparable to the controls. The significance of this reduction in glycogen concentration and its possible relationship to salicylate-induced foetal damage are discussed.

Key-words: Salicylates – prenatal influence – liver glycogen – abnormalities, drug-induced.

The disappearence of glycogen is one sign of liver ischaemia (BERNELLI-ZAZZERA & GAJA 1964). Liver glycogen is reduced by environmental factors e. g. starvation, anoxia and various drugs (GOETZE & MÜLLER 1963; SAMORAJSKI *et al.* 1965; MÄENPÄÄ & RÄIHÄ 1968). Moreover, post partum resistance to anoxia is related to heart glycogen content of newborns (DAWES *et al.* 1959; SHELLEY 1969). Glucose given to rabbits during labour has been shown to prolong foetal heart activity under anoxic conditions (GELLI *et al.* 1968).

Many studies have shown that salicylates decrease the content of glycogen in the adult liver (LUTWAK-MANN 1952; SPROULL 1954; SMITH & SMITH 1966). A high incidence of foetal death was found in earlier st

when salicylate was given to the mother late in pregnancy (LARSSON & ERIKSSON 1966). Liver haemorrhage and necrosis have been induced in foetuses within 4 to 8 hours after a single salicylate injection during the late foetal period (ERIKSSON 1969).

The present study was undertaken to investigate whether the salicylate-induced foetal liver damage was correlated with a decrease in liver glycogen. Moreover, it was of special interest to study the salicylate-effect on the concentration of glycogen in the heart. A comparison was made between two strains of mice with different susceptibility to drug-induced foetal damage (LARSSON 1962b; LARSSON & ERIKSSON 1966).

Material and Methods

Pregnant primiparous mice of the A/Jax and CBA strains were used and maintained as described elsewhere (LARSSON 1962a). Females were mated overnight and the day on which a vaginal plug was observed was denoted as day zero of pregnancy.

The investigation was carried out as two separate studies. In the first, performed during the spring to the autumn of 1968, the effect of sodium salicylate on maternal and foetal liver glycogen was studied in A/Jax mice (table 1). From the autumn of 1968 to the autumn of 1969 the same experiment was carried out on the CBA strain (table 1). In the second experiment, during 1969, the effect of different doses of salicylate was studied in A/Jax mice (table 2). Heart glycogen determinations were done in the same mice (table 3).

On day 17 of gestation at 10 a. m. the animals were injected intramuscularly with sodium salicylate 10 mg or 3 mg/20 g body weight in 0.1 ml distilled water (tables 1–3). At 4, 8 or 24 hours after injection the animals were sacrificed. The foetuses were removed and the number of dead was recorded as well as superficial and liver haemorrhages. Early foetal resorptions, which had obviously occurred before treatment, were not recorded. Uninjected mice of the same gestational age served as controls.

Two small pieces of maternal liver were taken from the centre of the right lobe. As far as possible the whole liver was removed from all the foetuses. The livers were immediately frozen in acetone cooled by dry ice. The samples were stored in test tubes kept on dry ice until analyzed. The foetal hearts were then collected, frozen, and stored in the same way.

Glycogen isolation and determination was performed by a slightly modified method of SEIFTER *et al.* (1950). Liver samples of about 50 mg, and the foetal heart samples of 5 mg, were put into a test tube containing 1 ml of 30 % potassium hydroxide. The tubes were placed in a boiling water bath for 20 minutes. After immediate cooling, 4 ml of 95 % ethanol were added together with 0.5 ml of 5 % sodium sulphate (HANDEL 1965). This solution was then gently brought to boiling point. Following cooling the contents were centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant was decanted and the tubes dried upside down on filter paper. Anthrone reagent (E. Merck AG, Darmstadt, Germany) (0.2 % in sulphuric acid) was then added for reading in a Beckman B spectrophotometer at 620 μm. The glycogen content, given in μg/mg frozen tissue, was calculated from a glucose standard curve. Statistical analysis was performed by "Student's" *t*-test.

Results

Glycogen was present in fairly high amounts in the foetal mouse liver on day 17 of gestation. 24 hours later the glycogen content had increased threefold (tables 1 & 2). Early on day 17 the maternal liver contained more glycogen than the foetal liver but on day 18 the relationship was reversed. The foetal heart glycogen increased slightly during day 17 of gestation ($P < 0.01$, table 3). The glycogen content was much higher in the foetal heart than in the maternal heart, which had a glycogen content of 1–2 μg/mg of frozen tissue (unpublished data).

The maternal liver glycogen was significantly (A/Jax: $P < 0.01$; CBA: $P < 0.05$) reduced 4 hours after the injection of 10 mg/20 g sodium salicylate (table 1). After 8 and 24 hours the glycogen content no longer showed any evidence on the effects of salicylate treatment.

The liver glycogen in unaffected foetuses was also decreased following salicylate treatment (tables 1 & 2). This decrease was most pronounced ($P < 0.001$) in both strains 4 hours after the injection. This marked reduction was still present at 8 hours in the A/Jax strain.

In the CBA strain, however, the reduction was less pronounced at 8 hours, but still significant ($P < 0.05$). With the 3 mg dose of salicylate the liver glycogen content returned to normal levels after 8 hours. After 24 hours, values comparable to the controls were observed in all the treated groups.

Foetuses with haemorrhages, both superficial and in the liver, had a lower liver glycogen content than the unaffected foetuses of the same group after 8 hours ($P < 0.05$). This reduction was still significant ($P < 0.001$) in these foetuses 24 hours after treatment.

Foetal heart glycogen was decreased in foetuses with superficial and/or liver haemorrhages taken as one group (4 hours: $P < 0.05$; 8 hours: $P < 0.001$; 24 hours: $P < 0.01$) but was not decreased in unaffected foetuses (table 3).

Discussion

In this study it has been shown that sodium salicylate decreases foetal liver glycogen in the mouse and, to a lesser degree, foetal heart glycogen in the mouse with haemorrhage. Late in gestation, glycogen is accumulated in the foetal liver as shown by biochemical and histochemical methods in many species (SHELLEY 1961; PETERS *et al.* 1963; SHELLEY 1964; TURCHINI & MALET 1964). In the mouse traces of liver glycogen are shown by histochemical methods on day 16 of gestation (PETERS *et al.* 1963). Thereafter the liver glycogen concentration increases rapidly so that on day 18 it exceeds

Table 1.

Liver glycogen in A/Jax and CBA mice treated with *sodium* salicylate (10 mg/20 g) on day 17 of gestation.

Strain	Treatment	Time of sacrifice after inj. hours	No. of litters	No. of foetuses	No. of dead foetuses	Liver glycogen µg/mg frozen tissue: Mothers		Living foetuses: Unaffected		Superficial haemorrhage		Liver haemorrhage	
						No.	Mean ± S. E. M.	No.	Mean ± S. E. M.	No.	Mean ± S. E. M.	No.	Mean ± S. E. M.
A/Jax	–	0	5	32		5	47.7 ± 3.5	32	21.5 ± 1.3				
CBA	–	0	6	47		4	36.9 ± 2.3	43	18.8 ± 1.1				
A/Jax	–	4	5	35		5	25.4 ± 4.0	34	23.4 ± 1.2				
A/Jax	Salicylate	4	6	48		5	9.3 ± 1.3	24	7.1 ± 0.7	15	7.1 ± 0.9	4	3.6 ± 0.6
CBA	–	4	5	37		5	23.7 ± 2.2	33	19.8 ± 1.1				
CBA	Salicylate	4	5	38		4	14.3 ± 2.4	21	11.2 ± 1.4	17	10.7 ± 1.2		
A/Jax	–	8	5	30		5	21.0 ± 2.4	30	27.4 ± 1.8				
A/Jax	Salicylate	8	6	42	16	6	14.4 ± 3.0	13	12.6 ± 2.4	5	2.6 ± 0.6	7	1.1 ± 0.5
CBA	–	8	4	27		4	13.1 ± 1.9	23	19.5 ± 1.3				
CBA	Salicylate	8	6	40		5	16.0 ± 1.8	15	14.3 ± 1.5	14	4.8 ± 0.9	1	2.5
A/Jax	–	24	5	33		5	29.3 ± 4.4	31	62.6 ± 3.1				
A/Jax	Salicylate	24	5	38	19	5	34.3 ± 4.1	11	54.6 ± 1.7	3	26.9 ± 4.4	5	29.8 ± 6.7
CBA	–	24	5	37		5	36.6 ± 4.2	34	49.4 ± 3.4				
CBA	Salicylate	24	5	40	3	5	35.3 ± 2.4	34	51.4 ± 3.7				

Table 2.

Liver glycogen in A/Jax mice treated with different doses of sodium salicylate on day 17 of gestation.

Substance given i. m. in 0.1 ml/20 g	Time of sacrifice after inj. hours	No. of litters	No. of foetuses	No. of dead foetuses	Foetal liver glycogen µg/mg frozen tissue					
					Unaffected		Superficial haemorrhage		Liver haemorrhage	
					No.	Mean ± S. E. M.	No.	Mean ± S. E. M.	No.	Mean ± S. E. M.
–	0	3	20		19	17.1 ± 1.9				
–	4	3	23		20	17.6 ± 1.2				
Salicylate 3 mg	4	5	36		25	11.1 ± 0.7				
Salicylate 10 mg	4	4	31		14	7.0 ± 0.8	9	4.9 ± 0.4	5	3.3 ± 1.0
–	8	4	31		22	20.1 ± 1.6				
Salicylate 3 mg	8	5	37		33	19.0 ± 0.9				
Salicylate 10 mg	8	6	47	8	15	7.5 ± 1.3	17	1.8 ± 0.5	6	1.1 ± 0.2
–	24	3	26		26	57.9 ± 2.7				
Salicylate 3 mg	24	6	43		38	49.6 ± 2.4				
Salicylate 10 mg	24	5	33	8	18	51.3 ± 2.9	4	28.3 ± 2 6	2	13.7 ± 0.5

Table 3.

Heart glycogen in A/Jax mice treated with sodium salicylate (10 mg/20 g) on day 17 of gestation.

Treatment	Time of sacrifice after inj. hours	No. of litters	No. of foetuses	No. of dead foetuses	Foetal heart glycogen μg/mg frozen tissue					
					Unaffected		Superficial haemorrhage		Liver haemorrhage	
					No.	Mean ± S. E. M.	No.	Mean ± S. E. M.	No.	Mean ± S. E. M.
–	0	5	35		30	11.1 ± 0.5				
–	4	5	43		37	11.1 ± 0.4				
Salicylate	4	5	39		19	10.8 ± 0.7	8	9.3 ± 1.0	4	7.7 ± 1.4
–	8	5	39		29	13.0 ± 0.7				
Salicylate	8	6	47	8	15	11.6 ± 0.9	13	7.3 ± 0.7	6	7.9 ± 1.3
–	24	6	50		41	13.3 ± 0.5				
Salicylate	24	5	33	8	19	13.0 ± 0.6	2	8.4 ± 0.1	2	8.6 ± 1.4

the liver glycogen concentration of the mother (PETERS *et al.* 1963). The heart glycogen content has been shown to be high in the foetus in many other species but decreases shortly before birth (SHELLEY 1961). It has been suggested that anaerobic glycogenolysis of these stores especially in the heart increases the ability of newborns to survive during anoxia (HIMWICH *et al.* 1942; MOTT 1961). Moreover, a direct relation has been found between the heart glycogen content and the survival time in different species (DAWES *et al.* 1959). Newborn tissue is also said to be more resistent to injury than adult cells (DAWKINS 1960 & 1964) possibly partly because of its higher glycogen concentration. By increasing the glycogen stores, especially in the foetal heart, it has been possible to demonstrate a greater resistance to anaerobic conditions (GELLI 1968). On the other hand, certain drugs such as insulin and chlorpromazine (COREY 1935; SAMORAJSKI *et al.* 1965) have been shown to reduce the foetal liver glycogen content. The present study demonstrates that sodium salicylate is capable of reducing foetal liver glycogen. How this treatment of the mother affects the foetus during the hypoxia of birth process can only be the object of speculation.

Salicylates have long been known to decrease liver glycogen in adults (SPROULL 1954) but no data were available for the foetus. Many different mechanisms for salicylate action in adults have been discussed, such as an increased glycogenolysis mediated by adrenal medullary stimulation and a decreased rate of glycogen synthesis (SMITH & SMITH 1966). On the one hand, the synthesis of glycogen and the maturation of the foetal rat liver have been shown to depend on the presence of corticosteroids (FAVARD & JOST 1966). On the other hand, sodium salicylate given to rats late in pregnancy has been shown to result in increased activity of the foetal adrenal cortex (LEMAIRE & GROSJEAN 1966). This study however, gives no further lead to the underlying mechanism of glycogen reduction in the foetus.

It is interesting to recall that the decrease in liver glycogen is most pronounced in foetuses with haemorrhage. It has previously been suggested that the haemorrhage, and observed hypoprothrombinaemia, are the result of more general liver damage (ERIKSSON 1970).

With the lower dose of salicylate the reduction in glycogen content could only be demonstrated 4 hours after administration. A more moderate decrease in glycogen content was observed with the higher dose in the less susceptible CBA strain (ERIKSSON 1969). These observations would raise the assumption that a relationship exists between the long lasting reduction in foetal liver and heart glycogen concentration and subsequent permanent foetal damage. It can be speculated that a decrease in glycogen concentration is probably a more sensitive indication of the foetal toxicity of a drug than the observation of morphological derangements.

Acknowledgement

I would like to take this opportunity of expressing my sincere gratitude to Drs. K. S. Larsson and H. Boström for their valuable advice and help during the investigation and to Mr. Kenneth Berglund for advice on statistical analysis. I also wish to thank Miss Gunhild Brolin for skilful technical assistance and Mrs. Inger Louthander for excellent secretarial help.

These studies were supported by grants from the Swedish Medical Research Council (B70-14X-993-05), Sempers Fond för Näringsforskning, Stockholm, Sweden, the Association for the Aid of Crippled Children, New York, U. S. A., and Främjande av ograduerade forskares vetenskapliga verksamhet, Karolinska institutet, Stockholm, Sweden.

REFERENCES

Bernelli-Zazzera, A. & G. Gaja: Some aspects of glycogen metabolism following reversible or irreversible liver ischemia. *Exp. Mol. Path.* 1964, **3**, 351–368.

Corey, E. L.: Fetal carbohydrate metabolism following adrenalectomy, insulin and glucose experiments on the mother. *Amer. J. Physiol.* 1935, **113**, 450–454.

Dawes, G. S., J. C. Mott & H. J. Shelley: The importance of cardiac glycogen for the maintenance of life in foetal lambs and newborn animals during anoxia. *J. Physiol. (Lond.).* 1959, **146**, 516–538.

Dawkins, M. J. R.: Autolytic changes in foetal liver. *J. Path. Bact.* 1960, **79**, 289–294.

Dawkins, M. J. R.: Cell injury in the newborn animal. *Cellular Injury. Ciba Symp.* 1964, 106–115.

Eriksson, M.: Salicylate-induced foetal haemorrhage in two mouse strains. *Acta path. microbiol. scand.* 1969, **76**, 164–170.

Eriksson, M.: Effect of salicylate treatment on fetal and maternal prothrombin time in the mouse. *Acta physiol. scand.* 1970, **78**, 39–42.

Favard, P. & A. Jost: Différenciation et charge en glycogène de l'hépatocyte du foetus de rat normal ou décapité. *Arch. Anat. micr. Morph. exp.* 1966, **55**, 603–632.

Gelli, M. G.: Prolongation of foetal heart activity during anoxia by glucose infusion in the mother animal before delivery. *Acta obstet. gynec. scand.* 1968, **47**, suppl. 2.

Gelli, M. G., G. Enhörning, E. Hultman & J. Bergström: Glucose infusion in the pregnant rabbit and its effect on glycogen content and activity of foetal heart under anoxia. *Acta paediat. scand.* 1968, **57**, 1–6.

Goetze, E. & G. Müller: Einfluss von Hunger und Insulin auf den foetalen Kohlenhydratstoffwechsel der Ratten an den letzten Trächtigkeitstagen. *Acta biol. med. germ.* 1963, **10**, 106–112.

Handel, E. van: Estimation of glycogen in small amounts of tissue. *Analyt. Biochem.* 1965, **11**, 256–265.

Himwich, H. E., A. O. Bernstein, H. Herrlich, A. Chesler & J. F. Fazekas: Mechanisms for the maintenance of life in the newborn during anoxia. *Amer J. Physiol.* 1942, **135**, 387–391.

Larsson, K. S.: Studies on the closure of the secondary palate. III. Autoradiographic and histochemical studies in the normal mouse embryo. *Acta morph. neerl.-scand.* 1962a, **4**, 349–367.

Larsson, K. S.: Studies on the closure of the secondary palate. IV. Autoradiographic and histochemical studies of mouse embryos from cortisone-treated mothers. *Acta morph. neerl.-scand.* 1962b, **4**, 369–386.

Larsson, K. S. & M. Eriksson: Salicylate-induced foetal death and malformations in two mouse strains. *Acta paediat. scand.* 1966, **55**, 569–576.

Lemaire, R. & O. Grosjean: Action du salicylate sodique sur le système hypophyso-surrénalien du rat au stade foetal et durant les premiers jours de la vie. *Rev. franc. Étud. clin. biol.* 1966, **11**, 696–706.

Lutwak-Mann, C.: The effect of salicylate and cinchophen on enzymes and metabolic processes. *Biochem. J.* 1942, **36**, 706–728.

Mott, J. C.: The ability of young mammals to withstand total oxygen lack. *Brit. med. Bull.* 1961, **17**, 144–147.

Mäenpää, P. H. & N. C. R. Räihä: Effects of anoxia on energy-rich phosphates, glycogen, lactate and pyruvate in the brain, heart and liver of the developing rat. *Ann. Med. exp. Fenn.* 1968, **46**, 306–317.

Peters, V. B., G. W. Kelly & H. M. Dembitzer: Cytologic changes in fetal and neonatal hepatic cells of the mouse. *Ann. N. Y. Acad. Sci.* 1963, **111**, 87–103.

Samorajski, T., J. M. Ordy & C. Rolsten: Prenatal chlorpromazine effects on liver enzymes, glycogen, and ultrastructure in mice offspring. *Amer. J. Path.* 1965, **47**, 803–831.

Seifter, S., S. Dayton, B. Novic & E. Muntwyler: The estimation of glycogen with the anthrone reagent. *Arch. Biochem.* 1950, **25**, 191–200.

Shelley, H. J.: Glycogen reserves and their changes at birth and in anoxia. *Brit. med. Bull.* 1961, **17**, 137–143.

Shelley, H. J.: Carbohydrate reserves in the newborn infant. *Brit. med. J.* 1964, **1**, 273–275.

Shelley, H. J.: The metabolic response of the fetus to hypoxia. *J. Obstet. Gynaec. Brit. Cwlth.* 1969, **76**, 1–15.

Smith, M. J. H. & P. K. Smith: *The salicylates.* John Wiley and Sons, Inc., New York. 1966.

Sproull, D. H.: The glycogenolytic action of sodium salicylate. *Brit. J. Pharmacol.* 1954, **9**, 121–124.

Turchini, J. P. & P. Malet: Glycogène et glucose-6-phosphatase dans le foie néonatal (souris) note histochimique. *Ann. Histochim.* 1964, **9**, 331–336.

Acta pharmacol. et toxicol. 1971, 29, 250–255.

From the Laboratory of Teratology and Department of Paediatrics,
Karolinska Institutet, Stockholm, Sweden

Salicylate-Induced Foetal Damage During Late Pregnancy in Mice: A Comparison between Sodium Salicylate, Acetylsalicylic Acid and Salicylsalicylic Acid

By

Margareta Eriksson

(Received September 25, 1970)

Abstract: Three salicylate derivatives i. e. sodium salicylate, acetylsalicylic acid and salicylsalicylic acid were tested and compared in A/Jax mice with regard to their effect on foetal damage on day 17 of gestation. The substances were administered by stomach tube in a dose constituting ⅔ of the LD50 dose in adult mice. Twentyfour hours after administration of each of the three derivatives an increased incidence of foetal death and haemorrhages were found. The highest incidence was found in the acetylsalicylic acid treated group. Four hours after administration all the salicylate derivatives caused a reduction in foetal liver glycogen. The control suspension however, also significantly reduced the glycogen content. The results are discussed in relation to the recommendations for drug testing. The advantage of comparing different drug derivatives for the study of the underlying mechanism responsible for their pharmacological actions is emphasized.

Key-words: Salicylates – prenatal influence – foetal death – liver glycogen.

Various derivatives of salicylates have been shown to induce malformations in several species when administered during the organogenetic period (Larsson 1970). Most reviews on the foetal hazards of drugs given during late pregnancy caution against the deleterious effect of salicylates (Apgar 1964; Yaffe 1968). However, only a few case reports are available in support of this opinion (Jackson 1948; Earle 1961). Recent studies from this laboratory have shown that sodium salicylate, given to mice late in pregnancy, causes an increased incidence of foetal death and haemorrhage and a reduction in foetal prothrombin and glycogen (Eriksson 1970a, b & 1971).

Comparisons between the influence of different salicylate derivatives on various metabolic and physiological processes have been of value in the clarification of the pharmacological action of salicylate (Karler *et al.* 1968; Åberg & Larsson 1970). Studies on the mechanism involved in the terato-

genic effect of salicylate have correlated the degree of inhibition of sulphomucopolysaccharide synthesis with the rate of induced malformations in mice (LARSSON & BOSTRÖM 1965).

Recommendations for the testing of drugs for teratogenicity nowadays include guidelines for a separate study late in pregnancy (FDA 1966; WHO REPORT 1967). In order to evaluate the foetal effect of a drug late in pregnancy other parameters than those used following treatment in the organogenetic period must be considered. Some possible parameters have been studied and discussed in our investigations following sodium salicylate treatment late in pregnancy (ERIKSSON 1970b). The present study was undertaken to compare, with guidence from recommendations for drug tests, three commonly used salicylate derivatives i. e. acetylsalicylic acid (ASA), salicylsalicylic acid (SSA) and sodium salicylate (NaSA) with regard to their morphological and biochemical effect during late pregnancy.

Material and Methods

Pregnant primiparous mice of the A/Jax strain were used and kept as described elsewhere (LARSSON 1962). They were mated overnight and the day of vaginal plug was denoted as zero day of pregnancy. On day 17 of gestation a single administration of the test substance suspended in 1 % sodium carboxymethylcellulose (CMC) in a volume of 0.2 ml/20 g bodyweight was given by stomach tube. The concentration of the substances administered was for sodium salicylate 66.6 mg/ml, acetylsalicylic acid 80 mg/ml, and salicylsalicylic acid 66.6 mg or 80 mg/ml. (The substances were kindly prepared by AB Bofors Nobel-Pharma and delivered as fresh solutions once a week). The doses were chosen as ⅓ of the LD50 dose, except for the higher SSA dose (determined at AB Bofors Nobel-Pharma, personal communication, G. ABERG 1970). Control animals were given only CMC.

The animals were sacrificed 4 or 24 hours after treatment (tables 1 & 2). Observations were made of premature delivery. The living foetuses were examined for superficial, liver or gastric haemorrhage Early foetal resorption, which had evidently occurred before treatment of the mother, is not included in the tables.

In the groups of animals killed 4 hours after treatment at least 5 foetuses from each litter were taken for liver glycogen determination (table 2). Untreated animals of the same age were used as controls. Glycogen was determined as described in previous paper (ERIKSSON 1971).

Statistical analysis was performed by "Student's" t-test.

Results

All the salicylate derivatives studied caused an increased incidence in foetal death (table 1). ASA showed a significantly higher incidence ($P < 0.001$) than NaSA and SSA. NaSA in turn had a higher frequency than

Table 1.

The morphological effect in A/Jax mice of different salicylate derivatives 24 hours after treatment on day 17 of gestation.

Substance	No. of litters	No. of foetuses	Dead foetuses		No. of living foetuses with		
			No.	%	superficial haemorrhage	liver haemorrhage	gastric haemorrhage
Carboxymethylcellulose 1 %	20	155	0	0	0	0	0
Sodium salicylate 13.3 mg/20 g	20	152	72	47	6	1	2
Acetylsalicylic acid 16 mg/20 g	20	134	104	78	9	1	0
Salicylsalicylic acid 13.3 mg/20 g	20	151	42	28	9	5	6
Salicylsalicylic acid 16 mg/20 g	20	135	52	39	18	11	3

Table 2.

Foetal liver glycogen in A/Jax mice 4 hours after treatment with different salicylate derivatives on day 17 of gestation.

Substance	No. of litters	No. of foetuses	Foetal liver glycogen µg/mg frozen tissue					
			Unaffected		*Superficial* haemorrhage		Liver haemorrhage	
			No.	Mean ± S. E. M.	No.	Mean ± S. E. M.	No.	*Mean* ± *S. E. M.*
Control	5	34	28	20 3 ± 1.1				
Carboxymethylcellulose 1 %	5	31	30	13.8 ± 1.1				
Sodium salicylate 13.3 mg/20 g	5	39	25	9.6 ± 0.9	13	8.1 ± 1.1		
Acetylsalicylic acid 16 mg/20 g	5	44	20	8.7 ± 0.9	17	7.8 ± 0.7	4	3.8 ± 0.5
Salicylsalicylic acid 13.3 mg/20 g	5	32	23	9.3 ± 0.8	5	6.8 ± 1.1	3	2.9 ± 1.0
Salicylsalicylic acid 16 mg/20 g	6	42	18	7.0 ± 1.0	21	4.6 ± 0 6	2	2.2 ± 0.8

the lower dose of SSA ($P < 0.001$). In the living foetuses superficial, liver and gastric haemorrhages, as described previously, were found in varying numbers (table 1) (ERIKSSON 1969 & 1970b). One female from each of the groups treated with NaSA, ASA and SSA 16 mg/20 g delivered before dissection on day 18 of gestation (not included in table 1).

Treatment with the control suspension of CMC produced a significant reduction in foetal liver glycogen ($P < 0.001$) (table 2). Treatment with NaSA, ASA and SSA further reduced the foetal liver glycogen ($P < 0.01$), SSA 16 mg/20 g ($P < 0.001$). No difference in the degree of glycogen reduction produced could be found between the derivatives. Foetuses with haemorrhages had a lower liver glycogen content than unaffected foetuses of the same group after treatment with SSA 16 mg/20 g ($P < 0.05$).

Discussion

In this study a comparison was made between the teratogenic effects of sodium salicylate, acetylsalicylic acid and salicylsalicylic acid. They all caused an increased rate of foetal death, foetal haemorrhage and a reduction in foetal liver glycogen.

This study was made in accordance with recommendations for drug testing late in pregnancy (FDA 1966). Thus, administration by stomach tube was used. It is interesting to note that by this route of administration, results similar to those after intramuscular injection of NaSA were obtained (ERIKSSON 1969 & 1970b). Furthermore the dose was a subtoxic one, corresponding to ⅔ of LD50 (ERIKSSON 1970b). The evaluation of foetal damage was based on the experience gained from earlier studies.

It is interesting to observe in this study that the administration of the control suspension of CMC alone caused a significant reduction in foetal liver glycogen although no macroscopic morphological injury could be found. Whether this was due to the stress-situation of feeding through a gastric tube (mediated to the foetus) or to a specific action of CMC can only be speculation. Following treatment with the different salicylate derivatives no correlation could be found between the reduction in foetal liver glycogen at 4 hours and the morphological damage found at 24 hours. A possible explanation could be that the glycogen content had already reached a minimum with the lower dose of SSA.

ASA has previously been shown to be less teratogenic than NaSA in the same mouse strain as used in this study (LARSSON & BOSTRÖM 1965). However, these substances were given in the same dose and not in a dose in relation to their LD50. It was suggested that the difference was due to different degrees of inhibition of mucopolysaccharide synthesis (LARSSON &

Boström 1965). *In vitro* studies have shown that SSA inhibits the synthesis of mucopolysaccharides to a greater extent than ASA (Åberg & Larsson 1970). In the present study, however, SSA has been shown to cause less foetal damage.

Pharmacokinetic studies in man have shown that ingestion of NaSA results in a high initial plasma concentration of salicylate while SSA ingestion and, to a lesser degree, ASA ingestion gives a low but long-lasting plasma concentration of salicylate (Nordqvist *et al.* 1965). This difference has also been shown in mice for NaSA and ASA (Ljungberg *et al.* 1968). ASA is rapidly hydrolyzed to salicylic acid *in vivo* (Ljungberg *et al.* 1968; Rowland & Riegelman 1968). It has been shown recently that SSA is hydrolyzed significantly more slowly than ASA in human whole blood and plasma *in vitro* (Personal communication, L. Harthon & M. Hedström 1970). Whether a foetal toxic effect is exerted by the given substance or its hydrolysate salicylic acid is not known. Recent studies in our laboratory have shown a high radioactivity in the foetus after the administration of ^{14}C-labelled salicylic acid to the mother (Eriksson & Larsson 1971). More pharmacological work is needed to answer this and related questions.

Acknowledgement

I would like to take this opportunity to express my sincere gratitude to Dr. K. S. Larsson and Dr. G. Åberg for their valuable advice and help during the investigation, and Mr. Kenneth Berglund for advice on statistical analysis. I also wish to thank Miss Gunhild Brolin for skilful technical assistance and Mrs. Inger Louthander for excellent secretarial help.

These studies were supported by grants from The Swedish Medical Research Council (14X-993-06), Expressen's Prenatal fond, Stockholm, Sweden, and the Association for the Aid of Crippled Children, New York, U. S. A.

REFERENCES

Åberg, G. & K. S. Larsson: Pharmacological properties of some antirheumatic salicylates. *Acta pharmacol. et toxicol.* 1970, **28**, 249–257.

Apgar, V.: Drugs in pregnancy. *J. Amer. med. Ass.* 1964, **190**, 840–841.

Earle, R.: Hazards to health. Congenital salicylate intoxication – Report of a case *New Engl. J. Med.* 1961, **265**, 1003–1004.

Eriksson, M.: Salicylate-induced foetal haemorrhage in two mouse strains. *Acta path microbiol. scand.* 1969, **76**, 164–170.

Eriksson, M.: Effect of salicylate treatment on fetal and maternal prothrombin time in the mouse. *Acta physiol. scand.* 1970a, **78**, 39–42.

Eriksson, M.: Salicylate-induced foetal damage during late pregnancy in mice. The modifying effect of repeated administration and dosage. *Acta paediat. scand.* 1970b, **59**, 517–522.

Eriksson, M.: The effect of salicylate on the glycogen content in foetal liver and heart in two strains of mice. *Acta pharmacol. et toxicol.* 1971, 29, in press.

Eriksson, M. & K. S. Larsson: Salicylate-induced foetal damage in two mouse strains. Studies on distribution of ^{14}C-labelled salicylic acid. *Acta pharmacol. et toxicol.* 1971, 29, in press.

Food and Drug Administration: Guidelines for reproduction studies for safety evaluation of drugs for human use. U. S. A., 1966.

Jackson, A. V.: Toxic effects of salicylate on the foetus and mother. *J. Path. Bact.* 1948, 60, 587–593.

Karler, R., W. C. Petty & T. S. Sulkowski: Some comparative biochemical effects of 5-substituted congeners of salicylic acid. *Arch. int. Pharmacodyn.* 1968, 173, 270–280.

Larsson, K. S.: Studies on the closure of the secondary palate. III. Autoradiographic and histochemical studies in the normal mouse embryo. *Acta morph. neerl.-scand.* 1962, 4, 349–367.

Larsson, K. S.: Action of salicylate on prenatal development. In: *Congenital malformations of mammalia.* Ed.: H. Tuchmann-Duplessis, Masson & Cie, Paris, 1970, in press.

Larsson, K. S. & H. Boström: Teratogenic action of salicylates related to the inhibition of mucopolysaccharide synthesis. *Acta paediat. scand.* 1965, 54, 43–48.

Ljungberg, S., U. Otto & L. Paalzow: Analgesic activity of acetylsalicylic and salicylic acid and its relation to blood concentrations in mice. *Acta pharm. suec.* 1968, 5, 489–500.

Nordquist, P., J. G. L. Harthon & R. Karlsson: Omsättningskinetik för salicylsalicylsyra, acetylsalicylsyra och natriumsalicylat hos människa. *Nord. Med.* 1965, 28, 1074–1076.

Principles for the testing of drugs for teratogenicity. Report of a WHO scientific group. *Pharm. Industrie* 1967, 29, 759–763.

Rowland, M. & S. Riegelman: Pharmacokinetics of acetylsalicylic acid and salicylic acid after intravenous administration in man. *J. pharm. Sci.* 1968, 57, 1313–1319.

Yaffe, S. J.: Foetal and neonatal toxicity of drugs. *Canad. med Ass. J.* 1968, 98, 301–306.

Acta pharmacol. et toxicol. 1971, 29, 256–264.

From the Laboratory of Teratology, Karolinska institutet,
Stockholm, Sweden

Salicylate-Induced Foetal Damage in Two Mouse Strains: Studies on the Distribution of ^{14}C-Labelled Salicylic Acid

By

Margareta Eriksson and K. Sune Larsson

(Received October 3, 1970)

Abstract: The distribution of salicylate was studied in pregnant and non-pregnant mice of the A/Jax and CBA strains. These strains were selected because of their different susceptibility to salicylate-induced foetal damage. Labelled salicylic-1-^{14}C-acid (1 μci) was injected intramuscularly together with 10 mg sodium salicylate as cold carrier in a volume of 0.1 ml distilled water/20 g bodyweight. The animals were sacrificed at different intervals from half an hour to 8 hours after the injection and the radioactivity in the maternal blood and liver and in the foetuses was determined by liquid scintillation counting. The non-pregnant mice showed initially a higher radioactivity in the blood than in the females on day 14 or 17 of gestation. The amount of liver radioactivity was similar in pregnant and non-pregnant animals and remained practically unchanged during the period of study. The foetal radioactivity was relatively high compared to that in the maternal liver. No strain difference were observed either on gestation day 14 or on day 17. The foetal radioactivity did not decrease during the first 4 hours after salicylate treatment on day 17 of gestation at which time foetal damage had already been produced.

Key-words: ^{14}C-salicylic acid – drug distribution – foetus – abnormalities drug-induced.

It is now well-known that foetal susceptibility to damage caused by exogenous agents differs, even between strains of the same species (Fraser *et al.* 1957; Larsson & Eriksson 1966). This is true not only for disturbances during the organogenetic period but also during the late stages of pregnancy (Eriksson 1969). The reason for these strain differences is not completely understood, although genetic studies have provided valuable information. The induced foetal damage, moreover, can be modified by additional exogenous factors (Gibson & Becker 1968). Pretreatment with a small dose of salicylate or with narcotic doses of pentobarbital (mebumalum NFN) for only two days, decreases the incidence of salicylate-induced foetal damage late in pregnancy, as shown in a recent study from our laboratory (Eriksson 1970).

Labelled compounds have been used in teratological experiments for investigating the metabolism of different embryonic tissues (LARSSON 1962a) and in studies on the distribution of teratogenic substances in the foetus as well as in the mother (WILSON *et al.* 1963; HANNGREN & ULLBERG 1963; KORANSKY & ULLBERG 1964; WADDELL & MARLOWE 1969). Tracer techniques have also been used in studies designed to elucidate the mechanism underlying strain differences in teratogenic susceptibility. Thus, with ^{14}C-cortisone LEVINE *et al.* (1968) demonstrated a slower elimination of isotope from foetuses of the A/Jax strain, with a high frequency of cortisone-induced cleft palate, than from foetuses of the resistant CBA strain (FRASER *et al.* 1957; LARSSON 1962a).

The A/Jax mice have been shown to be more susceptible than the CBA mice to salicylate-induced foetal damage (LARSSON & ERIKSSON 1966; ERIKSSON 1969). It was therefore of interest to use these two strains in the present study with ^{14}C-labelled salicylic acid in order to determine whether there were strain differences in the maternal and foetal handling of salicylate. This technique was also used to disclose changes in the maternal-foetal distribution of salicylate late in pregnancy after pretreatment with small doses of salicylate or of narcotic doses of pentobarbital, which decrease the frequency of salicylate-induced foetal damage.

Material and Methods

Adult non-pregnant female mice of the A/Jax strain and pregnant primiparous mice of the A/Jax and CBA strains were used and kept as described in previous studies from our laboratory (LARSSON 1962b). The day when a vaginal plug was observed was denoted as day zero of pregnancy. The labelled salicylic-1-^{14}C-acid (8.1 mg) (New England Nuclear Co., Boston, Mass., U.S.A.) was dissolved in 25 ml of sodium salicylate solution (100 mg/ml distilled water). The labelled salicylate was injected intramuscularly in a volume of 0.1 ml/20 g body weight corresponding to 1 μci labelled salicylic acid and 10 mg cold carrier. The animals were divided into the following experimental groups Non-pregnant A/Jax mice were sacrificed 30, 120 or 240 minutes after the injection of labelled salicylate (table 1). In a second experiment, both A/Jax and CBA mice received labelled salicylate on day 14 of gestation and were sacrificed after 30 or 240 minutes (table 2). When the isotope was given to females of the two strains on day 17 of gestation they were sacrificed after 30, 60, 120, 240 or 480 minutes respectively (table 3). In the last experiment A/Jax mice were pretreated with unlabelled sodium salicylate (3 mg/20 g intramuscularly) or with pentobarbital, nembutal®, (1.5 mg/20 g intraperitoneally) on day 15 and 16 of gestation. They were then injected with the labelled salicylate on day 17 of gestation and sacrificed after 30, 60, 120 or 240 minutes (table 4). All the injections were given at 10 a.m. and the animals were sacrificed by dislocation of the atlas after the above mentioned intervals.

Before sacrifice, duplicate blood samples of 25 μl were taken by retro-orbital puncture from the females and placed in Packard counting vials. A piece of liver of about

300 mg wet weight was taken from the adult animals. The number of implanted and living foetuses was recorded. After 4 and 8 hours on day 17 of gestation special observation for superficial haemorrhage was made as described elsewhere (ERIKSSON 1969). Not more than five foetuses were taken for the isotope measurements and they were weighed individually.

The liver sample and the foetuses were separately homogenized in 100 per cent ethanol in a Potter-Elvehjem homogenisator. The homogenate was transferred quantitatively to Erlenmeyer flasks and evaporated in an oven at 60°. The dry powder was digested in 2.5 ml of 70 per cent perchloric acid and 5 ml of a 30 per cent solution of hydrogen peroxide at 60° for 1 hour in the capped flasks (MAHIN & LOFBERG 1966). After cooling to room temperature three aliquots of 0.3 ml of the digested solution were transferred to Packard counting vials and 10 ml of scintillation counting fluid (1,000 ml toluene, 1,000 ml 2-methoxymethanol and 4 g omnifluor (New England Nuclear Co., Boston, Mass., U. S. A.) was added. The blood samples were digested in 0.1 ml of 70 per cent perchloric acid and 0.2 ml of a 30 per cent solution of hydrogen peroxide under the conditions described above and 10 ml of scintillation counting fluid was added. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer.

Results

With the technique used a high reproducibility in the results was obtained for the duplicate blood samples ($\sigma = 1339$). There was also minimal variation in the radioactivity between the different aliquots from the same liver or foetal homogenates.

In the non-pregnant mice little variability occurred among the values for blood or liver radioactivity (table 1). The non-pregnant mice showed a higher radioactivity in the blood than the females at two different stages of pregnancy (tables 2 & 3). Between 30 minutes and 4 hours after the injection of the isotope there was a linear fall in blood radioactivity in non-pregnant

Table 1.

Radioactivity in non-pregnant adult A/Jax mice following intramuscular injection of ^{14}C-salicylic acid.

No. of females	Time of sacrifice after inj. minutes	Blood cpm/25 µl Mean ± S. E. M.	Liver cpm/mg wet weight Mean ± S. E. M.
5	30	20499 ± 499	74 ± 3
5	120	17206 ± 1494	66 ± 2
4	240	12652 ± 328	66 ± 3

mice to about 60 per cent of the initial value. The mean liver radioactivity was in the same range as the individual values in the pregnant animals. There was practically no change in liver radioactivity during the period studied (table 1) which was also true for all the other groups, irrespective of treatment (tables 2, 3 & 4).

In day 14 pregnant mice – in spite of great variability in blood radioactivity – a tendency was observed for a decrease in the radioactivity between 30 minutes and 4 hours after injection (table 2). The CBA strain generally showed higher values than the A/Jax strain. Foetal radioactivity was initially slightly higher in the CBA strain but was equal to the radioactivity in the A/Jax strain after 4 hours.

In day 17 pregnant mice the blood radioactivity was also very variable. Nevertheless the blood radioactivity decreased slowly during the first 4 hours in both strains (table 3). The reduction was greater during the following 4 hours and after 8 hours only 20–30 per cent of the initial activity remained. No strain difference was observed. The radioactivity in the foetuses was

Table 2.

Radioactivity in A/Jax and CBA mice on gestation day 14 following intramuscular injection of ^{14}C-salicylic acid.

Strain	Time of sacrifice after inj. minutes	Maternal blood cpm/25 µl	Maternal liver cpm/mg wet weight	Foetus cpm/mg wet weight Mean[1] ± S. E. M.
A/Jax	30	15199	53	41 ± 3
		7291	49	31 ± 2
		7167	61	41 ± 2
	240	8564	57	33 ± 1
		7431	73	46 ± 2
		4787	61	41 ± 3
		4532	75	42 ± 2
CBA	30	19132	70	55 ± 4
		6687	55	48 ± 2
		17358	60	59 ± 3
	240	9971	61	48 ± 5
		11713	50	47 ± 2
		10043	47	35 ± 1

[1] Representing 5 foetuses in each litter.

Table 3.

Radioactivity in A/Jax and CBA mice on gestation day 17 following intramuscular injection of ^{14}C-salicylic acid.

Strain	Time of sacrifice after inj. minutes	Maternal blood cpm/25 µl	Maternal liver cpm/mg wet weight	Foetus cpm/mg wet weight		
				Unaffected Mean[2] ± S. E. M.	With haemorrhage Mean[2] ± S. E. M.	Dead Mean[2] ± S. E. M.
A/Jax	30	16113	49	35 ± 1		
		17247	78	37 ± 3		
		12858	–	35 ± 2		
		14497	44	32 ± 2		
	60	15783	76	38 ± 1		
		14688	69	35 ± 3		
		12888	–	43 ± 3		
		18113	63	44 ± 4		
	120	12433	66	44 ± 3		
		10289	56	32 ± 1		
		10032	60	39, 36, 41		
	240	–	63	39, 37, 40	37, 53	
		10671	67	38, 37, 32		
		9972	74	45 ± 3	49	
	480	3355	39		28, 25	25, 21
		4480	49			21, 21, 20
		4569	56		25	26 ± 1
		4428	56			25 ± 1
CBA	30	18588[1]	89	58 ± 7		
		18155[1]	70	64 ± 4		
		13836	74	40 ± 3		
		14571	63	44 ± 4		
	60	12566	64	46 ± 2		
		14647	79	43 ± 2		
		13403	65	50 ± 4		
	120	13264	71	43 ± 4		
		–	74	50 ± 2		
		12110	67	38 ± 2		
		13310	69	41 ± 1		
	240	–	67	39 ± 3		
		10584	63	47, 39, 47	35, 35	
		11433	79	40 ± 1	55	
		12913	60	32 ± 2		
	480	2597	45			32 ± 2
		3770	52		26 ± 3	
		3653	43	19	22	27
		3062	42	15, 17	16, 18	21

1 Single observation.

2 representing 4 or 5 foetuses in each litter; if less individual values are given.

unchanged during the first few hours in the A/Jax strain. In the CBA strain the foetal radioactivity was initially higher but decreased to that of the A/Jax level after 4 hours. After 8 hours the radioactivity was reduced to about half the initial value in both strains. No difference with regard to foetal radioactivity between the unaffected foetuses, foetuses with haemorrhage and dead foetuses were observed at 4 or 8 hours.

Table 4.

Radioactivity in A/Jax mice on gestation day 17 pretreated with sodium salicylate or pentobarbital following intramuscular injection of ^{14}C-salicylic acid.

Pretreatment	Time of sacrifice after inj. minutes	Maternal blood cpm/25 μl	Maternal liver cpm/mg wet weight	Foetus cpm/mg wet weight: Unaffected Mean[1] ± S. E. M.	Foetus cpm/mg wet weight: With haemorrhage Mean[1] ± S. E. M.
Sodium salicylate 3 mg/20 g, gestation days 15, 16	30	14125	72	33 ± 2	
		17006	63	45 ± 1	
		8740	55	33 ± 1	
	60	13244	60	39 ± 2	
		12947	61	40 ± 1	
		16079	58	42 ± 2	
	120	10089	58	34 ± 1	
		10476	58	39 ± 3	
		12087	68	50 ± 2	
	240	7910	52	26, 27, 28	24, 25
		12699	61		30, 35, 42
		8769	54	36 ± 2	30
Pentobarbital 1.5 mg/20 g, gestation days 15, 16	30	9053	57	37 ± 2	
		8804	80	48 ± 3	
		19676	49	34 ± 3	
	60	12675	81	58 ± 4	
		17562	58	49 ± 2	
		12394	57	39 ± 2	
	120	6268	69	44 ± 3	
		14080	73	37 ± 2	
		13107	53	30, 36	
	240	15165	62	36	38 ± 2
		14430	53	30, 43	23, 30, 32
		11266	44	35 ± 2	
		7853	48	29, 37	

[1] Representing 4 or 5 foetuses in each litter; if less individual values are given.

Pretreatment with a low dose of sodium salicylate or pentobarbital (mebumalum NFN) increased the variability in blood radioactivity between animals in the same group (table 4). The radioactivity in the blood as well as in the foetuses did not change during the first 4 hours studied. These values did not differ markedly from the values in the control group without pretreatment (table 3).

Discussion

With the technique used it has been possible to demonstrate a great individual variation in the handling of ^{14}C-labelled salicylic acid in pregnant mice.

The results obtained in the present study do not support the assumption that differences in the maternal-foetal distribution of salicylate might be responsible for the earlier reported strain differences in foetal damage, as demonstrated for cortisone by LEVINE *et al.* (1968). Moreover, changes in salicylate distribution can hardly explain the protective effect against the salicylate-induced foetal damage late in pregnancy by pretreatment with small doses of salicylate or narcotic doses of pentobarbital (ERIKSSON 1970).

It has been shown that high concentrations of ^{14}C-labelled salicylate or labelled metabolites are retained in the liver for a long period after intraperitoneal injection into adult male mice (STURMAN *et al.* 1968). In the present study the liver radioactivity was almost constant and was not correlated with the blood values or influenced by drug pretreatment.

It is interesting that the radioactivity in the foetuses is relatively high as compared to the radioactivity in the maternal liver. The present study confirms the results of earlier studies showing that foetal damage is produced within 4 hours after salicylate treatment on day 17 of gestation (ERIKSSON 1969). It is important to observe that the radioactivity was the same at 8 hours in the two strains of mice with different susceptibilities to salicylate-induced foetal damage. Using the same strains LEVINE *et al.* (1968) demonstrated that the uptake of ^{14}C-cortisone was similar but that the A/Jax foetuses (cleft palate-sensitive) retained a significant amount of the drug during the first 2 hours after administration. They suggested that genetic differences in the metabolism and tissue binding of cortisone were responsible for the described differences in retention of the teratogenic drug. It is, therefore, interesting in this study to observe a tendency toward the reverse situation with a somewhat initially lower foetal activity in the susceptible A/Jax strain. This was observed during the organogenetic period (day 14) as well as during the late foetal period (day 17).

Treatment with barbituric acid derivatives has been shown to give a more

rapid elimination of drugs or other potentially toxic substances in both adult and foetal animals (CONNEY 1967; CATZ & YAFFE 1968). No evidence has been obtained that this is the case following pretreatment with a low dose of salicylate or narcotic doses of pentobarbital, which protect against salicylate-induced foetal damage (ERIKSSON 1970). In this study neither faster elimination nor a reduced blood or foetal tissue radioactivities were observed. One must, however, be aware that the technique, as used by us and other investigators mentioned here, is a measure of the total activity and does not distinguish between the active substance and that which is conjugated and detoxified.

Most kinetic studies of salicylates have been done in man (SMITH & SMITH 1966; LEVY 1965). The metabolism differs between man and dog (ALPEN *et al.* 1951) and there is reason to believe that the same is true for other species even though few data are available. This study has shown moreover that there is great variability in the same strain. In man low doses of salicylate give a first order kinetic elimination curve but a first and a zero order kinetics are obtained with higher doses due to the saturation of salicyluric acid formation (LEVY 1965). In the mouse, salicyluric acid is formed but most of the salicylate is glucuronated (STURMAN *et al.* 1968). Another important factor in evaluating the effect of salicylate is the extent of its binding to plasma protein. Mice exhibit a low protein binding capacity for salicylate (STURMAN & SMITH 1967) which could result not only in higher toxicity but also faster elimination. Even for the intensively studied salicylates there is an apparent need for additional kinetic and metabolic studies to be made before the final evaluation of their foetal damaging effect.

Acknowledgement

We would like to take this opportunity to express our sincere gratitude to Miss Gunhild Brolin and Mrs. Inger van Moorselaar for their skilful technical assistance and Mrs. Inger Louthander for excellent secretarial help and Mr. Kenneth Berglund for help with the statistical analysis.

These studies were supported by grants from the Swedish Medical Research Council (B70-14X-993-05), the Association for the Aid of Crippled Children, New York, U. S. A., Expressen's prenatalfond, Stockholm, Sweden, and Främjande av ograduerade forskares vetenskapliga verksamhet, Karolinska institutet, Stockholm, Sweden.

REFERENCES

Alpen, E. L., H. G. Mandel, V. W. Rodwell & P. K. Smith: The metabolism of C^{14} carboxyl salicylic acid in the dog and in man. *J. Pharmacol. exp. Ther.* 1951, 102, 150–155.

Catz, C. & S. J. Yaffe: Barbiturate enhancement of bilirubin conjugation and excretion in young and adult animals. *Pediat. Res.* 1968, **2**, 361–370.
Conney, A. H.: Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* 1967, **19**, 317–366.
Eriksson, M.: Salicylate-induced foetal haemorrhage in two mouse strains. *Acta Path. Microbiol Scand.* 1969, **76**, 164–170.
Eriksson, M.: Salicylate-induced foetal damage during late pregnancy in mice. The modifying effect of repeated administration and dosage. *Acta paediat. Scand.* 1970, **59**, 517–522.
Fraser, F. C., B. E. Walker & D. G. Trasler: Experimental production of congenital cleft palate; genetic and environmental factors. *Pediatrics* 1957, **19**, 782–787.
Gibson, J. E. & B. A. Becker: Effect of phenobarbital and SKF 525–A on the teratogenicity of cyclophosphamide in mice. *Teratology* 1968, **1**, 393–398.
Hanngren, A. & S. Ullberg: Autoradiographic studies on the distribution of some chemotherapeutic agents. *IIIrd Internat. Congress of Chemotherapy*, Stuttgart, 1963, 710–720.
Koransky, W. & S. Ullberg: Autoradiographic investigations of C^{14}-labelled thalidomide and glutethimide in pregnant mice. *Proc. Soc. exp. Biol.* 1964, **116**, 512–516.
Larsson, K. S.: Closure of the secondary palate and its relation to sulpho-mucopolysaccharides. *Acta Odont. Scand.* 1962a, **20**, Suppl. 31.
Larsson, K. S.: Studies on the closure of the secondary palate. III. Autoradiographic and histochemical studies in the normal mouse embryo. *Acta Morph. Neerl. Scand.* 1962b, **4**, 349–367.
Larsson, K. S. & M. Eriksson: Salicylate-induced fetal death and malformations in two mouse strains. *Acta paediat. Scand.* 1966, **55**, 569–576.
Levine, A., S. J. Yaffe & N. Back: Maternal-fetal distribution of radioactive cortisol and its correlation with teratogenic effect. *Proc. Soc. exp. Biol.* 1968, **129**, 86–88.
Levy, G.: Pharmacokinetics of salicylate elimination in man. *J. pharm. Sci.* 1965, **54**, 959–967.
Mahin, T. D. & R. T. Lofberg: A simplified method of sample preparation for determination of tritium, carbon-14 or sulfur-35 in blood or tissue by liquid scintillation counting. *Analyt Biochem.* 1966, **16**, 500–509.
Smith, M. J. H. & P. K. Smith: *The salicylates.* John Wiley & Sons, New York, 1966.
Sturman, J. A. & M. J. H. Smith: The binding of salicylate to plasma proteins in different species. *J. Pharm. Pharmacol.* 1967, **19**, 621–623.
Sturman, J. A., P. D. Dawkins, N. McArthur & M. J. H. Smith: The distribution of salicylate in mouse tissues after intraperitoneal injection. *J. Pharm. Pharmacol.* 1968, **20**, 58–63.
Waddell, W. J. & C. Marlowe: The distribution of teratogenic agents in pregnant mice. *Teratology* 1969, **2**, 273.
Wilson, J. G., T. H. Shepard & J. F. Gennaro: Studies on the site of teratogenic action of C^{14}-labeled trypan blue. *Anat. Rec.* 1963, **145**, 300.

Acta pharmacol. et toxicol. 1971, 29, 265–274.

From the Department of Pharmacology, Institute of Pharmacy,
University of Oslo, Norway

Inhibition of Carrageenin-Induced Rat Paw Oedema by Substances Causing a Reduction of Kininogen and Prekallikrein in Plasma

By

Kjell Briseid, Finn Cato Arntzen and Olav Kjell Dyrud

(Received July 7, 1970)

Abstract: The acute, anti-inflammatory effect of bradykinin, ellagic acid, histamine, padutin ®, serotonin, trasylol® and trypsin was tested against carrageenin-induced rat paw oedema, and the *in vivo* effects of the same substances on parameters of the plasma kinin system was also examined The substances were injected intraperitoneally and oedema was induced half an hour later. Groups of 5 test- and 5 control rats were used, and measurements of initial paw volumes and of volumes after 1, 2 and 3 hours were used for calculating the inhibition values. All the substances tested, except padutin, inhibited the development of oedema and except for padutin and trasylol, caused a fall in total kininogen. Apart from bradykinin and padutin, the substances also reduced the amount of prekallikrein. The kininogen fractions activated by rat plasma kallikrein and by rat urine kallikrein were determined in order to calculate the 3 kininogen fractions $S1^{I}$, $S1^{II}$ and S2 (Briseid *et al.* 1970a). All the substances tested caused a fall in the amount of $S1^{II}$ and only trypsin in the amount of $S1^{I}$, while none of the substances altered the amount of S2. A significant correlation between the effects on factors involved in the plasma kinin system and the anti-oedema effects provides evidence for the presence of a causal relationship.

Key-words. Kinins – kininogen – kallikrein – carrageenin – rat paw oedema.

In some reports in the literature substances are mentioned capable of inducing rat paw oedemas on local application, and also capable of inhibiting the development of such oedemas when administered systemically in advance. Franchimont *et al.* (1961) found that serotonin oedema was inhibited by serotonin injected intraperitoneally, and Horáková & Muratová (1965) showed that serotonin, histamine and bradykinin given parenterally inhibited the oedemas caused by the same substances Garcia Leme *et al.* (1966) obtained similar results using the macromolecular substances carrageenin and cellulose sulphate. Most interesting was the observation of Horáková &

MURATOVÁ (1965) and also of GARCIA LEME *et al.* (1966) that each of the different irritants they used also protected against oedemas induced by the other irritants. This cross-resistance suggests a general mechanism which underlies the anti-inflammatory effects, and we therefore decided to examine if the inhibitory effects of serotonin, histamine and bradykinin on rat paw oedema were correlated with alterations in the plasma kinin system. The effect of trypsin, ellagic acid, padutin® (hog pancreas kallikrein) and trasylol® was also examined. Carrageenin, which had been found to cause a release of bradykinin when added to rat plasma (ROTHSCHILD & GASCON 1966), was chosen as the oedema-inducing agent, and the previously published methods for the determination of rat plasma prekallikrein and kininogen fractions were used (BRISEID *et al.* 1970b).

Technique

A. Materials and Assays.

Source of rats. Male Wistar albino rats (body weight 170–225 g) were obtained from the National Institute of Public Health, Oslo.

Rat plasma, rat plasma kininogen, rat plasma kallikrein and *rat urine kallikrein* were prepared as described previously (BRISEID *et al* 1970b).

Reagents. Bradykinin. Bradykinin, Sandoz A. G., Basel, Switzerland, was used as the standard substance in the assays. Bradykinin triacetate, Sigma Chem. Co., St. Louis, Missouri, U. S. A. was used for the *in vivo* experiments. *Carrageenin*, Marine colloids Inc., Springfield, N. J., U. S. A. *Ellagic acid.* Ellagic acid, Sigma Chem. Co. St. Louis, Missouri, U. S. A. *Histamine.* Histamine di-HCl, Light & Co., Ltd., Colnbrook, England. *Padutin*® (hog pancreas kallikrein), Bayer, A. G., Leverkusen, Germany. *Serotonin* 5-hydroxytryptamine creatinine sulphate, Hoffmann-la Roche & Co., Ltd. Basel, Switzerland. *Trasylol*® (protease inhibitor), Bayer, A. G., Leverkusen, Germany. *Trypsin.* Trypsin, twice cryst., washed free of salt, TRSF, Worthington Biochemical Corp, Freehold, New Jersey, U. S. A.

Assays. The kinin determinations were carried out on the isolated rat uterus as "bracketing assays", with a standard dose ratio 3 : 2. Bradykinin was used as standard

B. Methods.

Determination of factors of the kinin system. Determination of the kininogen fraction activated by incubation with plasma kallikrein, the kininogen fraction activated by urine kallikrein, total kininogen by incubation with (1) plasma kallikrein and (2) urine kallikrein and determination of rat plasma prekallikrein were as described previously (BRISEID *et al.* 1970b).

Rat paw oedema test. 0.1 ml of a 1 % (w/v) suspension of carrageenin in 0.9 % sodium chloride solution was injected through a 0.35 mm × 15 gauge needle into the plantar tissue of the right hind paw of an unanaesthetized rat. Immediately before the injection, the volume of the paw was measured, using a differential volume measuring instrument (U. Basile, Milan, Italy). Measuring was then carried out after 1, 2 and 3 hours respectively, and the average value was compared with the initial volume in order to estimate the per cent increase. For each inhibition experiment, a number of 10 rats were used divided at random into one control group and one test group. To the 5

Table 1.

Inhibition of carrageenin-induced rat paw oedema by serotonin injected intraperitoneally. Precision of the method.

Intraperitoneal injection: Serotonin in saline, 1 ml/100 g (0.1 % w/v).
Paw injection: Carrageenin suspension in saline, 0.1 ml/rat (1 % w/v).
30 minutes between intraperitoneal and paw injection.
Series I: 5 + 5 rats in each experiment.
Series II: Series I rats and 3 + 3 rats in each experiment.
For further details see text.

Series	Experi-ment	Intraperitoneal injection	Per cent increase in rat paw volume after time in hours				Inhibition per cent
			1	2	3	Average	
I	1	Serotonin	31	28	45	35	24
		No injection	34	44	59	46	
	2	Serotonin	25	30	41	32	18
		No injection	22	42	54	39	
	3	Serotonin	32	36	54	41	24
		No injection	31	56	76	54	
	4	Serotonin	28	26	45	33	21
		No injection	21	41	63	42	
	5	Serotonin	30	34	52	39	19
		No injection	31	50	63	48	
II	1	Serotonin	39	33	51	41	16
		No injection	37	48	63	49	
	2	Serotonin	24	29	42	32	22
		No injection	21	44	57	41	
	3	Serotonin	38	42	59	46	21
		No injection	35	58	80	58	
	4	Serotonin	30	27	43	33	18
		No injection	20	40	60	40	
	5	Serotonin	28	32	50	37	24
		No injection	29	50	67	49	
I	Mean ± S. D.						21.2 ± 2.8
II	Mean ± S. D.						20.2 ± 3.2

control rats a volume of saline corresponding to the volume of drug solution given to the test rats was administered intraperitoneally.

Comments on the technique.

Rat paw oedema test. Swelling of the carrageenin-injected right hind paw reached a peak in 3 to 4 hours, and then retained the same degree of oedema for at least 2 hours.

Table 2.

Effects on carrageenin-induced rat paw oedema of various substances injected intraperitoneally.

Number of rats in control and test groups: 5.
Intraperitoneal injections: 1 ml/100 g rat. (Ellagic acid 2 ml/100 g rat).
Paw injections: 0.1 ml/rat of a 1 % (w/v) suspension of carrageenin in saline.
For further details see text.

Experiment number	Intraperitoneal injection	Time in minutes before carrageenin	Per cent increase in rat paw volume after time in hours				Inhibition per cent
			1	2	3	Average	
1	Trypsin, 25 mg/kg	30	7	11	16	11.3	59
	Saline	–	17	26	40	27.7	
2	Trypsin, 25 mg/kg	120	13	19	28	20.0	43
	Saline	–	15	39	51	35.0	
3	Padutin®, 100 KU/kg[1]	30	24	34	53	37.0	4
	Saline	–	25	36	55	38.7	
4	Serotonin, 10 mg/kg	30	7	8	20	11.7	51
	Saline	–	13	20	39	24.0	
5	Trasylol®, 100000 KIU/kg[2]	45	5	10	24	13.0	43
	Saline	–	12	22	34	22.7	
6	Bradykinin, 2.5 mg/kg	30	10	22	39	23.7	24
	Saline	–	13	38	43	31.3	
7	Bradykinin, 5.0 mg/kg	30	16	18	33	22.3	34
	Saline	–	31	31	41	34.0	
8	Bradykinin, 2.5 mg/kg	60 *after*	12	23	35	23.3	9
	Saline	–	16	25	36	25.7	
9	Histamine, 25 mg/kg	7	8	11	28	15.7	16
	Saline	–	5	15	36	18.7	
10	Histamine, 25 mg/kg	30	6	13	35	18.0	28
	Saline	–	10	19	46	25.0	
11	Ellagic acid, 0.6 mg/kg	30	7	26	32	21.7	37
	Saline	–	15	36	52	34.3	
12	Ellagic acid, 0.6 mg/kg	120	12	27	39	26.0	29
	Saline	–	22	26	52	36.7	
13	Ellagic acid, 0.6 mg/kg	60 *after*	20	46	55	39.7	49 (increase)
	Saline	–	14	26	40	26.7	

[1]) KU = Kallikrein units.
[2]) KIU = Kallikrein inactivator units

The average volume of measurements at 1, 2, and 3 hours was accordingly chosen for the calculations. The volume of the saline-injected left hind paw was also measured after 1, 2, and 3 hours respectively. No significant alterations could, however, be observed during this period, and hence the calculations of the carrageenin-induced

Table 3.

Effects on total kininogen and prekallikrein in rat plasma of various substances injected intraperitoneally.

Intraperitoneal injections: 1 ml/100 g rat. (Ellagic acid 2 ml/100 g rat).
Total kininogen activated by rat plasma kallikrein followed by rat urine kallikrein. The batches of kallikrein preparations were tested for their kinin releasing activity in comparison with a standard batch.
For further details see text.

Kininogen and prekallikrein batch number	Number of rats	Intraperitoneal injection	Time in minutes before blood collection	Kinin released per ml plasma as bradykinin		Prekallikrein test/ standard
				µg	%	
1–7	90	–	–	2.0	100	1.0
8	7	Trypsin, 25 mg/kg	10	1.3	65	0.7
9	8	Trypsin, 25 mg/kg	30	1.1	55	0.4
10	11	Trypsin, 25 mg/kg	120	1.8	90	0.9
11	7	Padutin®, 100 KU/kg	30	2.0	100	0.8
12	8	Serotonin, 10 mg/kg	30	1.7	85	0.6
13	7	Trasylol®, 10^5 KIU/kg	45	1.8	90	0.4
14	5	Bradykinin, 1.5 mg/kg	30	1.5	75	0.9
15	6	Bradykinin, 1.5 mg/kg	120	1.7	85	1.0
16	7	Histamine, 25 mg/kg	30	1.5	75	0.7
17	7	Histamine, 25 mg/kg	120	1.7	85	0.8
18	7	Ellagic acid, 0.6 mg/kg	30	1.5	75	0.9
19 + 20	7 + 8	Ellagic acid, 0.6 mg/kg	120	1.8	90	0.6

volume increases were based on the initial measurement of the same paw.

The day to day variations in carrageenin-induced increases in paw volume were sometimes quite considerable, and it was considered important to test a group of control rats in parallel

The precision of the method was tested with serotonin, a substance which only exerts a moderate inhibiting effect under the conditions used. Table 1 shows the results of experiments carried out both with groups of 5 and with groups of 8 rats over a period of 2 months. The reproducibility was considered satisfactory. In the precision experiments the intraperitoneal injection of saline to the control rats, which was routinely carried out in all the other experiments, was omitted. The results can accordingly not be compared with the results shown in table 2.

Results

Table 2 shows the effects of various substances on carrageenin-induced rat paw oedema. All the substances tested inhibited, to a varying extent, the development of oedema except for hog pancreas kallikrein (padutin®).

Table 4.

Effects on kininogen fractions in rat plasma of various substances injected intraperitoneally.

I. The kininogen fraction activated by an excess amount of rat plasma kallikrein preparation.
II. The kininogen fraction activated by an excess amount of rat urine kallikrein preparation.
III. Total kininogen activated by rat plasma kallikrein and rat urine kallikrein.

The hypothetical fractions S1I and S2 were calculated as the differences between the data in columns III and II and in III and I respectively. The remaining kininogen fraction was thus S1II (BRISEID *et al.* 1970a).

For further details see text.

Kininogen batch number	Number of rats	Intraperitoneal injection	Time in minutes before blood collection	Kinin released per ml plasma as μg bradykinin					
				I	II	III	S1I	S1II	S2
1–7	90	–	–	1.5	1.4	2.0	0.6	0.9	0.5
8	7	Trypsin, 25 mg/kg	10	0.8	1.1	1.3	0.2	0.6	0.5
9	8	Trypsin, 25 mg/kg	30	0.7	0.9	1.1	0.2	0.5	0.4
10	11	Trypsin, 25 mg/kg	120	1.2	1.2	1.8	0.6	0.6	0.6
11	7	Padutin®, 100 KU/kg	30	1.4	1.0	2.0	1.0	0.4	0.6
12	8	Serotonin, 10 mg/kg	30	1.0	1.2	1.7	0.5	0.5	0.7
13	7	Trasylol®, 10^5 KIU/kg	45	1.2	1.2	1.8	0.6	0.6	0.6
14	5	Bradykinin, 1.5 mg/kg	30	1.1	1.1	1.5	0.4	0.7	0.4
15	6	Bradykinin, 1.5 mg/kg	120	1.2	0.9	1.7	0.8	0.4	0.5
16	7	Histamine, 25 mg/kg	30	1.1	0.9	1.5	0.6	0.5	0.4
17	7	Histamine, 25 mg/kg	120	1.2	0.9	1.7	0.8	0.4	0.5
18	7	Ellagic acid, 0.6 mg/kg	30	0.9	0.8	1.5	0.7	0.2	0.6
19 + 20	7 + 8	Ellagic acid, 0.6 mg/kg	120	1.2	1.0	1.8	0.8	0.4	0.6

ellagic acid was given during the development of oedema instead of before the carrageenin injection, an *increase* in oedema was observed. When bradykinin was given at the same point of time no significant effect on the oedema was seen.

Table 3 shows the results of determinations of the total kininogen and the prekallikrein in plasma obtained from rats treated with the same substances as had been tested for their rat paw oedema inhibiting effects. All the substances tested caused a fall in total kininogen except for the protease inhibitor trasylol® and hog pancreas kallikrein (padutin®), but the effect of serotonin was not clearly significant (BRISEID *et al.* 1970b). However, the effect of serotonin on the kininogen fraction activated by plasma kallikrein (table 4) was very marked.

In addition it should be mentioned that even if padutin did not alter the total kininogen, the enzyme markedly reduced the kininogen fraction activated by rat urine kallikrein (table 4).

Taking into consideration the precision of the method and the variation between groups of rats (BRISEID *et al.* 1970b) all the substances tested except padutin and bradykinin caused a reduction in the level of prekallikrein in plasma.

In addition to the total kininogen, table 4 shows the amounts of kininogen activated by rat plasma kallikrein and by rat urine kallikrein. These data were then used to calculate the amounts of the hypothetical kininogen fractions $S1^{I}$, $S1^{II}$ and S2 (BRISEID *et al.* 1970a). According to this paper plasma kallikrein releases kinin from 2 kininogen fractions, $S1^{I}$ and $S1^{II}$, while urine kallikrein releases kinin from the 2 kininogen fractions $S1^{II}$ and S2. Table 4 shows that trypsin reduced the amount of both fraction $S1^{I}$ and $S1^{II}$, while the other substances tested seemed to alter only the amount of fraction $S1^{II}$. S2 was not significantly influenced by any of the substances tested. (One of the substances, padutin®, significantly increased the $S1^{I}$ fraction, while $S1^{II}$ was correspondingly reduced.) It should be emphasized, however, that the error of the data, obtained indirectly as difference values, are so considerable that only pronounced differences from the control material should be considered.

Discussion

The kinin system in the rat paw oedema test. Several research workers have produced evidence for the participation of the kinin system in inflammatory reactions, and the rat paw oedema test has frequently been used as a model. Kinins or kinin-like substances were demonstrated in fluid collected from rat paw oedema induced by thermal influence (ROCHA E SILVA & ANTONIO 1960; STARR & WEST 1967), serotonin (BONTA & DE VOS 1965 &

Table 4.

Effects on kininogen fractions in rat plasma of various substances injected intraperitoneally.

I. The kininogen fraction activated by an excess amount of rat plasma kallikrein preparation.
II. The kininogen fraction activated by an excess amount of rat urine kallikrein preparation
III. Total kininogen activated by rat plasma kallikrein and rat urine kallikrein.

The hypothetical fractions $S1^{I}$ and S2 were calculated as the differences between the data in columns III and II and in III and I respectively. The remaining kininogen fraction was thus $S1^{II}$ (Briseid *et al.* 1970a).

For further details see text.

Kininogen batch number	Number of rats	Intraperitoneal injection	Time in minutes before blood collection	Kinin released per ml plasma as µg bradykinin					
				I	II	III	$S1^{I}$	$S1^{II}$	S2
1–7	90	–	–	1.5	1.4	2.0	0.6	0.9	0.5
8	7	Trypsin, 25 mg/kg	10	0.8	1.1	1.3	0.2	0.6	0.5
9	8	Trypsin, 25 mg/kg	30	0.7	0.9	1.1	0.2	0.5	0.4
10	11	Trypsin, 25 mg/kg	120	1.2	1.2	1.8	0.6	0.6	0.6
11	7	Padutin®, 100 KU/kg	30	1.4	1.0	2.0	1.0	0.4	0.6
12	8	Serotonin, 10 mg/kg	30	1.0	1.2	1.7	0.5	0.5	0.7
13	7	Trasylol®, 10^5 KIU/kg	45	1.2	1.2	1.8	0.6	0.6	0.6
14	5	Bradykinin, 1.5 mg/kg	30	1.1	1.1	1.5	0.4	0.7	0.4
15	6	Bradykinin, 1.5 mg/kg	120	1.2	0.9	1.7	0.8	0.4	0.5
16	7	Histamine, 25 mg/kg	30	1.1	0.9	1.5	0.6	0.5	0.4
17	7	Histamine, 25 mg/kg	120	1.2	0.9	1.7	0.8	0.4	0.5
18	7	Ellagic acid, 0.6 mg/kg	30	0.9	0.8	1.5	0.7	0.2	0.6
19 + 20	7 + 8	Ellagic acid, 0.6 mg/kg	120	1.2	1.0	1.8	0.8	0.4	0.6

observed. Of particular interest was the observation that an intraperitoneal injection of ellagic acid *during* the development of oedema significantly added to the oedema volume. This effect might well have been caused by an immediate release of kinin in the blood.

The species unspecific kallikrein preparation padutin, which does not deplete rat plasma kininogen, had no anti-oedema effect. The polyvalent protease inhibitor trasylol did not influence the kininogen, but caused a low prekallikrein value, and a significant inhibition of the paw oedema.

Effects of the various substances tested on kininogen fractions. The species unspecific hog pancreas kallikrein (padutin®) did not alter the total kininogen, but reduced the $S1^{II}$-fraction and correspondingly increased the $S1^{L}$ fraction (table 4). This observation suggest an interrelationship between the two kininogen fractions, a finding which makes further investigations necessary. The fact that all the other substances tested which had an effect on the kininogen, apart from trypsin, only reduced the amount of the $S1^{II}$-fraction, leaving the $S1^{I}$-fraction apparently unchanged, does not necessarily mean that the first-mentioned fraction was directly influenced. A reduction in the amount of $S1^{I}$ followed by a conversion of $S1^{II}$ to $S1^{I}$ would lead to the same result. As mentioned previously it should be stressed that the data given in table 4 for the 3 hypothetical kininogen fractions are not very precise, since they were obtained indirectly. Only marked deviations from the control figures should be taken into consideration.

A comparison of the effects of serotonin, histamine and bradykinin, and also some other substances tested for their inhibition of carrageenin-induced rat paw oedema and for their effects on parameters of the kinin system, demonstrates a significant correlation between the two sets of examinations. This result provides evidence for the assumption that the anti-oedema effect of the above mentioned substances is due to an effect on the plasma kinin system.

REFERENCES

Bonta, I. L. & C. J De Vos: Presence of a slow-contraction inducing material in fluid collected from the rat paw oedema induced by serotonin. *Experientia* 1965, **21**, 34–38.

Bonta, I. L. & C. J. De Vos: Significance of the kinin system in rat paw oedemas and drug effects on it. *Europ. J Pharmacol.* 1967, **1**, 222–225.

Briseid, K., O. K. Dyrud & F. C. Arntzen: Substrates for kinin-releasing enzymes in rat plasma. *Acta pharmacol. et toxicol.* 1970a, **28**, 138–144.

Briseid, K., O. K. Dyrud & S. Øie: Determination of factors of the kinin system in rat plasma. *Acta pharmacol. et toxicol.* 1970b, **28**, 124–137.

Di Rosa, M. & L. Sorrentino. The mechanism of the inflammatory effect of carrageenin. *Europ. J. Pharmacol.* 1968, **4**, 340–342.

Franchimont, P., J. Lecomte & H. van Cauwenberge: Influence de la sérotonin et d'un de ses antagonistes sur le développement des réactions oedémateuses locales provoquées chez le rat par divers agents. *C. R. Soc. Biol.* 1961, **155**, 180–184.

Garcia Leme, J., E. S. Schapoval & M. Rocha E Silva: Factors influencing local oedema in the rat's paw produced by macromolecular agents (dextran, carrageenin and cellulosesulfate. In: *Abstracts. III International Pharmacological Congress*, Sao Paulo, 1966, 155.

Gilfoil, T. M. & I. Klavins: 5-hydroxytryptamine, bradykinin and histamine as mediators of inflammatory hyperesthesia. *Amer. J. Physiol.* 1965, **208**, 867–876.

Horáková, Z. & Muratová: Means of influencing the oedematous component of inflammation. In: *International symposium on non-steroidal anti-inflammatory drugs.* Eds. S. Garattini and M. N. G. Dukes, International Congress Series No. 82, Excerpta Medica Foundation, Amsterdam 1965, 237–244.

Rocha E Silva, M.: Pathogenesis of acute inflammation. I. Chemical Mediators. In: *Proceedings of the fourth panamerican congress of rheumatology.* International Congress Series No. 165, Excerpta Medica Foundation, Mexico City, 1967, 17–26.

Rocha E Silva, M. & A. Antonio: Release of bradykinin and the mechanism of production of a "thermic oedema" (45°C) in the rat's paw. *Med. exp.* 1960, **3**, 371–382.

Rothschild, A. M.: Some pharmacodynamic properties of cellulose sulphate, a kininogen-depleting agent in the rat. *Brit. J. Pharmacol.* 1968, **33**, 501–512.

Rothschild, A. M. & L. A. Gascon: Sulphuric esters of polysaccharides as activators of a bradykinin-forming system in plasma *Nature* 1966, **212**, 1364.

Schapoval, E., J. Garcia Leme & M. Rocha E Silva: Edema in the rat's paw induced by local injection of cellulosesulfate. In: *Abstracts. III International Pharmacological Congress*, Sao Paulo, 1966, 154.

Starr, M. S. & G. B. West: Bradykinin and oedema formation in heated paws of rats. *Brit. J. Pharmacol.* 1967, **31**, 178–187.

Van Arman, C. G., A. J. Begany, L. M. Miller & H. H. Pless: Some details of the inflammations caused by yeast and carrageenin (with appendix on kinetics of the reaction). *J. Pharmacol.* 1965, **150**, 328–334.

Acta pharmacol. et toxicol. 1971, **29**, 275–283.

From the Department of Pharmacology, Institute of Pharmacy,
University of Oslo, Norway

A Comparison of the Inhibition by Cardiac Glycosides of the Isolated Intestine from the Rat and the Guinea Pig

By

Gudrun Briseid and Kjell Briseid

(Received July 7, 1970)

Abstract: A method described by BIELTVEDT & BRISEID (1966) and BIELTVEDT (1967) for the determination of the inhibition by cardiac glycosides of the isolated, histamine-stimulated guinea pig ileum was modified to estimate the inhibition of the acetylcholine-stimulated rat jejunum. The glycoside concentrations causing 50 % inhibition of submaximal, isotonic contractions were determined, and the procedure was based on 20-minute contact periods between the muscle preparations and the cardioactive substances, and then 22-minute equilibration periods with chemical stimulation. The degree of inhibition was found to depend on the potassium content of the Tyrode solutions used. In a low potassium Tyrode solution (1.8 mM) the inhibition of the rat intestine by cardiac glycosides was found to be of the same order of magnitude as the inhibition of the guinea pig intestine. When the potassium chloride concentration was increased to 2.7 mM, the well known species difference was observed, the concentration of cardiac glycoside required for inhibition of the rat intestine being about 30 times higher than that required for inhibition of the guinea pig intestine. The range of potency of the different cardiac glycosides and aglycones which were tested in the low potassium Tyrode solution, was the same for the two species. Presupposing an inhibition of Na^+, K^+-activated transport ATPases as the basic mechanism for the inhibition by cardiac glycosides of the isolated intestine preparations, a theory of qualitative differences between the transport enzymes in the two species is advanced In the rat intestine the presence is suggested of one ATPase which is rather resistant to inhibition by cardiac glycosides, but sensitive to a reduction in potassium concentration, in addition to a glycoside-susceptible ATPase also present in the guinea pig intestine.

Key-words Digitalis glycosides – smooth muscle inhibition – species difference.

Recent studies indicate that there is no correlation between the considerable differences in species susceptibility to cardiac glycosides and the metabolism rate of the glycosides (HERRMANN & REPKE 1964a & b; HERRMANN *et al.* 1964). A clear parallelism was, however, pointed out for several species between the

inhibition by cardiac glycosides of the Na^{+}, K^{+}-activated membrane ATPases from heart muscle on the one hand, and the effect on isolated hearts or intact animals on the other (REPKE *et al.* 1965).

The low susceptibility to digitalis glycosides of the rat as compared with the guinea pig is well known. This species difference was also observed in experiments on isolated hearts (GERSMEYER & HOLLAND 1962; LÜLLMANN & HOLLAND 1962). In a previous investigation (BIELTVEDT 1967) the inhibition by a number of cardiac glycosides and aglycones of the isolated guinea pig ileum was determined. Similar experiments have now been carried out on the isolated rat intestine to examine if the marked species difference observed in isolated hearts was also detectable in the isolated intestine.

Technique

1. *Procedure.* The procedure described by BIELTVEDT & BRISEID (1966) for guinea pig ileum was used with the following modifications: Pieces of jejunum from fasted and freshly killed albino rats weighing 140–205 g were used. The load on the muscle was usually 1.4 g (range 0.8–1.6 g). Acetylcholine at concentrations of 40 to 320 ng/ml (modified Tyrode solution, see table 2) was used as agonist. Only one antagonist (cardiac glycoside or aglycone) was used for each piece of jejunum, out in increasing

Table 1.

Concentration ranges used of different cardiac glycosides and aglycones for the estimation of the inhibiting effects on the acetylcholine-stimulated, isolated rat jejunum. Modified Tyrode solution: See table 2.

Substance	Concentration range ng/ml
Verodoxin	70– 100
Gitaloxin	70– 140
Digitoxin	60– 200
Strophanthin-G	200– 340
Digoxin	300– 600
Digitoxigenin	500–1400
Lanatoside C	580–1570
Gitoxin	1000–1680
Digoxigenin	1200–4900
Digitalinum verum	2100–3500
Gitoxigenin	5000–9800

doses as described previously (BIELTVEDT & BRISEID 1966). This means that an experiment was considered to be finished when the concentration of antagonist causing a 50 % reduction of the submaximal, acetylcholine-induced contractions could be calculated. Each result was calculated on the basis of the average effect obtained with digitoxin, which was used as standard substance and frequently assayed over the experimental period. The average concentration of digitoxin causing a 50 % reduction of the agonist effect was 133 ng/ml (range 79–191 ng/ml).

2. *Glycoside and aglycone concentration.* Table 1 shows the concentration ranges required of the different cardioactive substances in a modified (see table 2) Tyrode solution. Each range is based on several experiments. For most of the substances stock solutions were made up in 96 % ethanol. The final alcohol concentration in the organ bath never exceeded 0.8 %. Gitaloxin, gitoxin and verodoxin were dissolved in a mixture of chloroform and methanol (1 + 1 v/v). The concentration of chloroform-methanol mixture in the final solutions never exceeded 0.2 ‰ for gitaloxin and verodoxin, and 0.2 % for gitoxin.

3. *Materials and Tyrode solutions.* Gitaloxin, verodoxin: Boehringer & Soehne, GmbH, Mannheim, Germany. Digitoxigenin, digitoxin, digoxigenin, digoxin, gitoxigenin, gitoxin: Fluka AG., Buchs, Switzerland. Digitalinum verum: Hoffmann-La Roche & Co. Ltd. Co., Basle, Switzerland. Strophanthin-G (g-Strophanthinum cryst., Ouabain): Merck A. G., Darmstadt, Germany. Lanatoside C: Sandoz, Ltd., Basle, Switzerland. Table 2 shows the Tyrode solutions used.

Comments on the technique

1. *Use of rat jejunum instead of rat ileum.* In order to make a comparison with previous experiments on the isolated guinea pig ileum (BIELTVEDT & BRISEID 1966; BIELTVEDT 1967) rat ileum should be used as the test organ in the present work. Preliminary experiments, however, showed that rat jejunum regularly gave more stable responses to agonist stimulation, and that the tendency to curvature of the dose effect curves was less pronounced than for the rat ileum preparations. As the concentrations of agonist required to cause submaximal contractions were the same, and as the concentrations of cardiac glycosides required for agonist inhibition were also roughly the same for the two parts of the rat intestine, the jejunum was chosen as the test preparation in the present work.

2. *Use of acetylcholine as agonist.* In the previous work on cardiac glycoside inhibition of the isolated guinea pig ileum (BIELTVEDT & BRISEID 1966; BIELTVEDT 1967) histamine was used as agonist. Because of the well known lack of response to histamine of the isolated rat intestine, acetylcholine was used as agonist in the present experiments. The use of acetylcholine should, however, not compromise the comparison between the two species. Preliminary experiments demonstrated that the concentrations of acetylcholine required for submaximal contractions were roughly the same for intestine preparations from the guinea pig and from the rat, and also that the inhibiting concentrations of digitoxin were of the same order of magnitude.

3. *Use of one antagonist only for each jejunum preparation.* In previous exper' with the isolated guinea pig ileum, a technique was adopted in which the

Table 2.

Tyrode solutions used.

	g substance per 100 ml						
Tyrode	NaCl	$CaCl_2$	KCl	$MgCl_2$	NaH_2PO_4	$NaHCO_3$	Glucose
Normal	0.80	0.02	0.02	0.01	0.005	0.10	0.10
Modified	0.81	0.01	0.013	"	"	"	"

washed free from the cardiac glycoside or aglycone by means of a Tyrode solution with increased potassium content (Bieltvedt & Briseid 1966). The muscle was then ready for contact with a new antagonist. Such a procedure allowed the testing of the standard glycoside, digitoxin, and another cardioactive substance in parallel on the same piece of intestine. In the present work a strongly reduced contraction to acetylcholine was regularly observed after inhibition of the rat intestine by a cardiac glycoside followed by the above mentioned washing procedure. A further assay of another antagonist could thus not be carried out. With regard to the low potassium content of the modified Tyrode solution (table 2) as the main basis for the depression of the rat intestine, see "Results" and "Discussion".

Results

Estimations of the rat jejunum inhibiting effects of 7 cardiac glycosides and 3 aglycones were carried out with digitoxin as standard substance. The results are shown in table 3 which also gives results taken from similar experiments carried out previously on the isolated guinea pig ileum (Bieltvedt 1967). It can be seen that there was a close correlation between the range of potencies of the cardioactive substances for the two species. The amounts of glycosides and aglycones required for 50 % inhibition were of the same order of magnitude, roughly twice as large concentrations being required for the inhibition of the rat jejunum than for inhibition of the guinea pig ileum. The only substance which markedly differed in potency in the two species was the glycoside gitoxin, a concentration of about 6 times higher being required for the inhibition of the rat intestine than for inhibition of the guinea pig intestine. It should be mentioned, however, that the gitoxin preparation previously used in the experiments on the guinea pig ileum was found by paper chromatography to contain as much as 15–20 % of the highly active digitoxin (Bieltvedt 1967). The inhibitory effect of the present gitoxin preparation was found to be almost the same on the guinea pig ileum and the rat jejunum.

All the experiments were carried out in a Tyrode solution modified to contain 66 % of the usual amount of potassium chloride, i. e. 1.8 mM

against 2.7 mM (BIELTVEDT & BRISEID 1966). Moreover the calcium chloride concentration was lower in the modified Tyrode, 50 % of the normal, and corresponded to 0.9 mM.

Experiments carried out with digitoxin as test substance in normal Tyrode solution showed that the decrease in inhibition caused by an increase in potassium content deviated markedly for the two species. While the amount of cardiac glycoside required for 50 % inhibition of the guinea pig intestine increased about 4 times (from about 70 to about 260 ng/ml), the amount required for the rat intestine increased about 60 times (from about 140 ng/ml to about 8 μg/ml). Fig. 1 shows a typical experiment with rat jejunum in normal Tyrode as well as in Tyrode solution 1.8 mM as against potassium chloride. It can be seen that a considerable digitoxin concentration was required for 50 % inhibition in the normal Tyrode, more than 5 μg/ml was necessary, while about 126 ng/ml sufficed in the modified Tyrode solution.

Table 3.

Inhibition by cardiac glycosides and aglycones of the isolated acetylcholine-stimulated rat jejunum and the histamine-stimulated guinea pig ileum.

Estimation of the inhibitory effects of 10 different substances, digitoxin being used as standard substance The average value of digitoxin causing 50 % inhibition was 133 ng/ml (12 experiments, range 79–191 ng/ml) for rat jejunum and 70 ng/ml (12 experiments, range 56–90 ng/ml) for guinea pig ileum.
The guinea pig ileum results were taken from a previous paper (BIELTVEDT 1967).
The Tyrode solution was modified to contain 66 % of the normal potassium and 50 % of the normal calcium
For further details see text and BIELTVEDT & BRISEID (1966).

Cardiac glycosides and aglycones	ng/ml causing 50 % inhibition mean values		Relative activity test/standard per cent, molar	
	Rat jejunum	Guinea pig ileum	Rat jejunum	Guinea pig ileum
Gitaloxin	106	43	134	159
Verodoxin	96	32	113	148
Strophanthin-G	285	160	46	50
Digoxin	629	230	21	33
Lanatoside C	983	495	18	15
Gitoxin	1613	245	9	20
Digitoxigenin	994	545	7	5
Digitalinum verum	3010	1950	5	4
Digoxigenin	2723	1585	3	4
Gitoxigenin	7404	3125	1	1

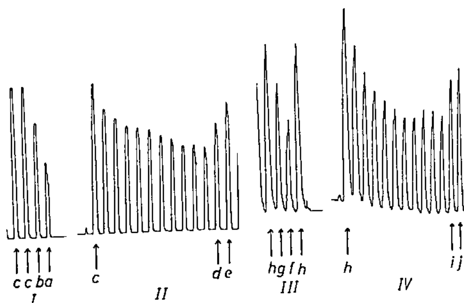


Fig. 1. Inhibitory effect of digitoxin on the isolated acetylcholine-stimulated rat jejunum.
Experiment 1: I, II. Modified Tyrode solution, 1.8 mM potassium chloride.
Experiment 2: III, IV. Normal Tyrode solution, 2.7 mM potassium chloride.
I, III. Acetylcholine effect before digitoxin inhibition.
II, IV. Acetylcholine effect after 20 minutes' contact between the muscle and digitoxin, II: 126 ng/ml, IV. 5 μg/ml.

Acetylcholine concentration.

a: 40 ng/ml	f: 40 ng/ml
b: 80 "	g: 60 "
c: 160 "	h: 80 "
d: 320 "	i: 120 "
e: 640 "	j: 160 "

The letters refer to the marked and the subsequent cycles.

Discussion

According to Godfraind (1964) the inhibitory action exerted by cardiac glycosides on chemically stimulated smooth muscle is due to the inhibition of active transport. Na^+, K^+-activated membrane ATPases, specifically inhibited by cardiac glycosides, have been detected in the intestinal epithelium of the guinea pig as well as of the rat (Taylor 1962; Berg & Chapman 1965; Berg & Szekerczes 1966), and it seems reasonable to suggest that the inhibition described in the present work on isolated intestine preparations from the rat and the guinea pig by cardiac glycosides, is due to the inhibition of such enzymes. In this case a pronounced species difference would be expected. The strong resistance to digitalis glycosides of the isolated rat heart, as compared

with the isolated heart from the guinea pig (GERSMEYER & HOLLAND 1962; LÜLLMANN & HOLLAND 1962), was also observed for preparations of Na^+, K^+-activated ATPase of heart muscle from the same species (REPKE *et al.* 1965), while the *in vivo* difference in toxicity of cardiac glycosides in the two species is well known. It might accordingly seem surprising that no significant difference in inhibition by cardiac glycosides was observed in the main series of experiments presented in this work, between intestinal preparations from the rat and the guinea pig. These experiments were, however, carried out in a Tyrode solution modified to contain a lower concentration than usual of potassium chloride (1.8 mM against 2.7 mM) and also of calcium chloride (0.9 mM against 1.7 mM). On the other hand, experiments carried out to examine the inhibition by digitoxin of the acetylcholine-stimulated rat intestine and guinea pig intestine in *normal* Tyrode solution (2.7 mM KCl and 1.7 mM $CaCl_2$) clearly demonstrated a marked species difference, 30 times higher concentrations of the cardiac glycoside being required for the inhibition of the rat tissue than for inhibition of the guinea pig tissue. As will be evident from the further discussion, the predominant reason for the elimination of the species difference in the modified Tyrode solution was the lowering of the potassium ion concentration, the decrease in calcium ion concentration contributing only insignificantly.

The reduction in the potassium ion concentration was originally carried out to increase to some extent the susceptibility of the guinea pig ileum to inhibition by cardiac glycosides, and to steepen the log glycoside-inhibition curves (BIELTVEDT & BRISEID 1966). The use of a Tyrode solution with low calcium ion concentration was, however, not introduced especially for the experiments mentioned above, but had been used routinely in our laboratory to eliminate spontaneous contractions of the guinea pig ileum which were otherwise sometimes troublesome. In the above mentioned work (BIELTVEDT & BRISEID 1966) it was demonstrated that the increase in sensitivity of the guinea pig ileum to digitoxin in the modified Tyrode solution was predominantly determined by the decrease in potassium ion concentration, the low calcium ion concentration being of less importance for the effect. Experiments carried out in the present work with isolated rat intestine in a Tyrode solution with 1) normal calcium chloride concentration (1.7 mM) and low potassium chloride concentration (1.8 mM) and 2) low calcium chloride concentration (0.9 mM) and low potassium chloride concentration (1.8 mM) gave results similar to those obtained on the isolated guinea pig ileum: The degree of inhibition by a cardiac glycoside was only slightly influenced by the change in calcium ion concentration.

The importance of the concentrations of sodium and potassium for the inhibition by cardiac glycosides of the Na^+, K^+-activated membrane ATPase is well known (DUNHAM & GLYNN 1961; BERG & SZEKERCZES 1966). In

comparative experiments on intestinal epithelium and red cells from rats the last mentioned authors pointed out that ouabain was a stronger inhibitor in assays of intestinal enzymes than in assays of red cell enzyme only because different potassium and sodium concentrations were used. With an increase in the potassium/sodium ratio the apparent difference between the two tissues disappeared. It seems probable that an inhibition of Na^+, K^+-activated membrane ATPases by the cardiac glycosides is the basic mechanism for the inhibition of the isolated intestinal preparations observed in the present work, and also that the reduction in potassium/sodium ratio eliminated the inherent difference in species susceptibility to the glycosides. In the Tyrode solutions commonly used the sodium chloride concentration is 137 mM and the potassium chloride concentration 2.7 mM, which means that the amount of sodium ion roughly corresponds to that of the extracellular fluid, while the amount of potassium ion is lower. The species difference was, however, first eliminated in the modified Tyrode solution in which the potassium ion concentration was further reduced to 1.8 mM. Thus *in vivo*, the Na^+, K^+-activated ATPases of the rat intestine will be only insignificantly inhibited by concentrations of cardiac glycosides which will markedly inhibit the intestinal enzymes of the guinea pig. REPKE *et al.* (1965) concluded from their experiments on the inhibition by ouabain of the transport ATPases from 4 species that the potassium antagonism was probably not the basis of the species differences. The potassium ion concentration was, however, only altered in the concentration range over 2.5 mM.

Different interpretations of the present results are possible, but the fact that a reduction in potassium concentration practically eliminated the species difference might indicate the presence in the rat intestine of one ATPase rather resistant to cardiac glycosides, but sensitive to a reduction in potassium concentration, – in addition to a glycoside susceptible ATPase which is also present in the guinea pig. Some evidence in support of such a theory was also provided by experiments in which intestinal preparations from the rat and from the guinea pig were continuously stimulated by submaximal acetylcholine concentrations in normal (2.7 mM) and in low potassium Tyrode (1.8 mM). While the contractions of the isolated guinea pig intestine were relatively unchanged for periods of at least 6 hours in both normal and in low potassium Tyrode, the contraction height of the rat intestine preparations remained stable in normal Tyrode, but declined relatively quickly (1 to 2 hours) when the low potassium Tyrode was used. In addition it should be mentioned that there are reports in the literature pointing to qualitative differences between the sodium/potassium pump mechanisms in the rat intestine as compared with the intestine in other species (ROBINSON 1967; 1969).

The range of inhibition registered in the low potassium Tyrode for cardio-

active glycosides and aglycones, and which was found to be the same for both species, might reflect their relative affinities to the tissues (GODFRAIND & LESNE 1968).

REFERENCES

Berg, G. G. & B. Chapman: The sodium and potassium activated ATPase of intestinal epithelium. I. Location of enzymatic activity in the cell. *J. Cell. Comp. Physiol.* 1965, **65**, 361–372.

Berg, G. G. & J. Szekerczes: The sodium and potassium activated ATPase. II. Comparative study of intestinal epithelium and red cells. *J. Cell. Comp. Physiol.* 1966, **67**, 487–500.

Bieltvedt, G.: Inhibition by cardiac glycosides and aglycones of the isolated guinea pig ileum: Estimation of relative potencies. *Acta pharmacol. et toxicol* 1967, **25**, 80–86.

Bieltvedt, G. & K. Briseid: The inhibitory action of cardiac glycosides on the isolated guinea pig ileum: Effects of alterations in the ionic composition of the medium. *Acta pharmacol. et toxicol.* 1966, **24**, 157–168.

Dunham, E. T. & I. M. Glynn: Adenosinetriphosphatase activity and the active movements of alkali metal ions. *J. Physiol.* 1961, **156**, 274–293.

Gersmeyer, G. & W. C. Holland: Effect of ouabain on Ca exchange in rat atria. *Pharmacologist* 1962, **4**, 164

Godfraind, T.: L'action biphasique des glucosides cardiotoniques. *Actual. Pharmacol.* 1964, **17**, 237–269.

Godfraind, T. & M. Lesne: Relative potency of digitoxin and digoxin in relation with their binding to isolated cardiac and smooth muscles. *Arch. int. Pharmacodyn.* 1968, **171**, 228–230.

Herrmann, I., H. J. Portius & K. Repke: Untersuchungen zur Ursache der Digitalisresistenz der Kröte *Arch. exp Path. u. Pharmak.* 1964, **247**, 1–18.

Herrmann, I & K Repke: Entgiftungsgeschwindigkeit und Kumulation von Digitoxin bei verschiedenen Species *Arch exp. Path u Pharmak.* 1964a, **247**, 19–34.

Herrmann, I & K. Repke. Epimerisierung und Hydroxylierung von digitaloiden Steroidlactonen durch Fermente der Leber. *Arch. exp. Path. u. Pharmak.* 1964b, **248**, 351–369.

Lüllmann, H. & W Holland: Influence of ouabain on an exchangeable calcium fraction, contractile force, and resting tension of guinea-pig atria. *J. Pharmacol. exper. Ther.* 1962, **137**, 186–192.

Repke, K., M. Est & H. J. Portius: Über die Speciesunterschiede in der Digitalisempfindlichkeit *Biochem Pharmacol.* 1965, **14**, 1785–1802.

Robinson, J. W L : The loss of intestinal transport capacity following preincubation in sodium-free media *in vitro Pflugers Archiv* 1967, **294**, 182–200.

Robinson, J W. L.: Species differences in the intestinal response to sulphate ions. *FEBS Letters* 1969, **5**, 157–160.

Taylor, C B: Cation-stimulation of an ATPase system from the intestinal mucosa of the guinea-pig. *Biochim. Biophys. Acta* 1962, **60**, 437–440.

Acta pharmacol. et toxicol. 1971, 29, 284-294.

From the Departments of Pharmacology and Gynaecology,
University of Oulu, Finland

Metabolism of Chlorpromazine and *p*-Nitrobenzoic Acid in the Liver, Intestine and Kidney of the Human Foetus

By

Olavi Pelkonen, Martti Vorne, Pentti Jouppila and Niilo T. Kärki

(Received August 10, 1970)

Abstract: The ability of the liver, intestine and kidney to metabolize chlorpromazine (CPR) and *p*-nitrobenzoic acid (NBA) was studied in the human foetus. Low levels of CPR metabolizing activity were present in all the tissues studied, but only the liver and intestine were capable of metabolizing NBA. We were not able to detect any cytochrome P-450 in the liver microsomal fraction. The enzymes metabolizing CPR and NBA are located in the liver microsomes, they require NADP and the 100,000 $\times$ g supernatant fraction or $NADPH_2$ for full activity, are inhibited by carbon monoxide, and have Michaelis constants of the same order of magnitude as found in the enzymes from experimental animals. The above mentioned characteristics of the foetal enzymes strongly suggest that they belong to the same class of $NADPH_2$-dependent mixed function oxidases which are detected in the livers of adult humans and animals and which are thought to be responsible for the greater part of oxidative and reductive drug metabolism.

Key-words: Foetus/enzymology – drugs/metabolism.

In general, foetal and newborn animals have a limited capacity to metabolize drugs (PARKE 1968). The microsomal enzymes of the liver which oxidatively metabolize drugs, including cytochrome P-450, are more or less absent in the foetus, but increase rapidly after birth in the rat, reaching adult levels at about 30 days (KATO *et al.* 1964). The reduction of nitro and azo compounds is also impaired in newborn animals (FOUTS 1962). Similarly, the ability of foetal tissues to synthesize conjugates of drugs and their metabolites is minimal or altogether absent in most instances, though on the other hand, acetylation and sulphate formation have been reported to be present in normal adult levels (VEST & ROSSIER 1963).

In a previous investigation we have determined the ability of human foetal tissues to oxidize chlorpromazine (CPR), hexobarbitone (enhexymalum NFN) and meperidine (pethidinum NFN), to reduce *p*-nitrobenzoic acid (NBA),

and to conjugate *p*-nitrophenol (PELKONEN *et al.* 1969). In the present paper we report more exact data on the metabolism of CPR and NBA by human foetal liver, intestine and kidney and some of the characteristics of the respective liver enzymes.

Material and Methods

Mothers and foetuses.

Foetuses were obtained from the Department of Gynaecology where legal abortions were performed. We have no exact data of the medication of mothers before the abortion. The method of abortion used was the so called minor section. The age of the mothers varied from 21 to 40 years. Most of them were in good health or the abortion was made because of psychiatric disease. The weight of the foetuses varied from 19 to 500 grams and their age ranged from 8 to 26 weeks as determined from the last menses of the mother.

Tissue preparations.

The whole liver, kidneys and the small intestine were excised and homogenized in four volumes of 0.1 M potassium phosphate buffer, pH 7.4 with a Potter-Elvehjem homogenizer in the cold room (+2–+4°). Homogenates were centrifuged at 12,000 × g for 20 minutes and the supernatants were preserved and used for the experiments. In order to obtain microsomes and the "soluble" fraction, the 12,000 × g supernatant was centrifuged at 100,000 × g for 60 minutes with an MSE-50 ultracentrifuge. All the fractions were stored in the cold room at 0° unless the measurements were carried out immediately. Only those tissues which were excised within half an hour of the abortion were used for the experiments

Enzyme assays.

The incubation mixture contained 0.2 mmol of KCl, 0.01 mmol of $MgCl_2$, 0.006 mmol of glucose-6-phosphate, and 0.25 µmol of NADP. Under standard conditions the substrates used was as follows: CPR 0.078 and NBA 3.0 µmol. The enzyme preparation and 0.1 M potassium phosphate buffer, pH 7.4, were added so that the final volume was 4 ml. The incubation time was usually one hour, but this varied from 15 to 120 minutes in some experiments. The incubation was done in a metabolic shaker at 37° in atmospheric air for CPR and in a nitrogen atmosphere for NBA. In the latter case N_2 was used, because the formation of *p*-amino benzoic acid declines to about 80 % in the presence of air (FOUTS & BRODIE 1957). The substrate left (chlorpromazine) or the metabolic product (*p*-amino benzoic acid) was assayed according to the following methods: CPR by the method of SALZMAN & BRODIE (1956) and NBA according to FOUTS & BRODIE (1957). We made sure that under these incubation conditions there was sufficient NADP-reducing activity to prevent it from becoming the limiting factor for the reaction

Cytochrome P-450 was assayed by the method of SCHENKMAN *et al.* (1967).

Protein was assayed by the Biuret reaction.

Results

We were able to detect the disappearance of CPR in the li[illegible] and kidney 12,000 × g supernatants of human foetus. The d[illegible] CPR

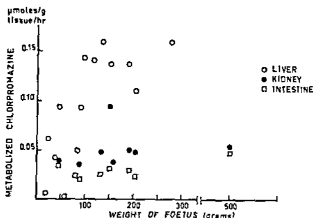


Fig. 1. Metabolism of chlorpromazine by foetal tissues as a function of foetal weight. Cofactor mixture is described in Material and Methods. The amount of substrate was 0 078 µmol, and the 12,000 × g supernatant was used as an enzyme source. Incubation time was one hour.

as a function of foetal weight is shown in fig. 1. The CPR-metabolizing activity in the liver of the foetuses weighing 50 grams or less varied from zero to 0.1 µmol per gram of tissue wet weight per hour. Foetuses of 100 grams or more, had CPR-metabolizing activity in all tests and the activity varied from 0.10 to 0.17 µmol per gram of tissue wet weight per hour. *In the intestine and kidney a trace of activity was found which did not increase with the increasing foetal weight.*

NBA-metabolizing activity was detected in the liver and intestine, but not in the kidney. Fig. 2 presents the results for NBA metabolism.

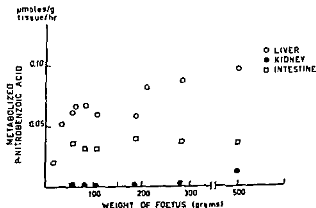


Fig. 2. Metabolism of *p*-nitrobenzoic acid by foetal tissues as a *function of foetal* weight. Cofactor *mixture is described in* Material and Methods. The amount of substrate was 3.0 µmol, and the 12,000 × g supernatant was used in determinations. Incubation time was one hour.

Table 1.

Effect of storage on the metabolism of chlorpromazine. Tissues were homogenized in 0.1 M potassium phosphate buffer pH 7.4, and centrifuged at 12,000 × g for 20 minutes. Whole tissues and 12,000 × g supernatants were stored in a cold room (+ 2° – + 4°) in ice before the experiments. Activities are expressed in micromoles of metabolized chlorpromazine per gram of tissue wet weight per hour and also in percents of the activity at the beginning of the experiment.

Tissue	Time of homogenisation and centrifugation after tissue preparation (days)	Time of determination of enzyme activity after homogenization (days)							
		0		1		3		4	
		μmol	%	μmol	%	μmol	%	μmol	%
Liver	0	0.14	100	0.15	107			0.12	86
	1	0.14	100			0.12	86		
	4	0.04	29						
Intestine	0	0.05	100					0.00	0
	1	0.04	80			0.00	0		
	4	0.01	20						
Kidney	0	0.05	100	0.03	60			0.00	0

Effect of storage on metabolism.

The results of the storage experiment are presented in table 1. The activity in the excised liver was preserved for at least one day, when it was stored in a cold room in ice. The 12,000 × g supernatant kept its activity for several days in a cold room in ice. The intestine and kidney preparations were much more labile as compared to the liver preparations kept in storage.

Metabolism of CPR as a function of time.

A typical experiment for CPR metabolism *in vitro* as a function of time is seen in fig. 3. The rate of metabolism of the liver was constant for up to one hour. The rate of metabolism in the intestine and kidney preparations slowed down in thirty minutes. The storage experiment also seems to indicate that metabolic activities in the intestine and kidney are more labile than the activity in the liver and that they become inactivated sooner under the experimental conditions.

Cofactor requirements of enzyme systems.

Table 2 presents the cofactor requirements of the CPR and NBA metabolizing enzymes. NADP and the 100,000 × g supernatant were necessary for full activity. The absence of $MgCl_2$ reduced the activity to ⅓–½ of the stan-

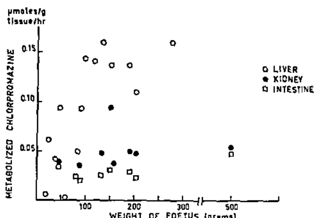


Fig. 1. Metabolism of chlorpromazine by foetal tissues as a function of foetal weight. Cofactor mixture is described in Material and Methods. The amount of substrate was 0.078 μmol, and the 12,000 × g supernatant was used as an enzyme source. Incubation time was one hour.

as a function of foetal weight is shown in fig. 1. The CPR-metabolizing activity in the liver of the foetuses weighing 50 grams or less varied from zero to 0.1 μmol per gram of tissue wet weight per hour. Foetuses of 100 grams or more, had CPR-metabolizing activity in all tests and the activity varied from 0.10 to 0.17 μmol per gram of tissue wet weight per hour. In the intestine and kidney a trace of activity was found which did not increase with the increasing foetal weight.

NBA-metabolizing activity was detected in the liver and intestine, but not in the kidney. Fig. 2 presents the results for NBA metabolism.

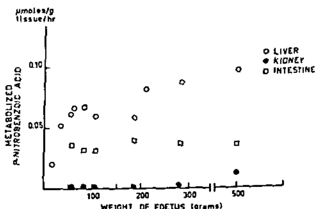


Fig. 2. Metabolism of *p*-nitrobenzoic acid by foetal tissues as a function of foetal weight. Cofactor mixture is described in Material and Methods. The amount of substrate was 3.0 μmol, and the 12,000 × g supernatant was used in determinations. Incubation time was one hour.

Table 3.

Cellular localization of enzyme activity. Various cell fractions were prepared from foetal liver by differential centrifugation and incubated with 0.078 μmol of chlorpromazine and 3.0 μmol of NBA. Cofactor mixtures is described in Material and Methods. Activities are expressed in micromoles of metabolized substrate per milligram of microsomal protein per hour except in B where the activity is expressed in micromoles per milligram of total protein per hour.

	Metabolized substrate μmol × 10^{-3}	
	CPR	NBA
A Whole homogenate	9.5	3.5
B 12,000 × g pellet fraction	0.2	0.0
C 12,000 × g supernatant fraction	12.5	4.5
D 100,000 × g pellet fraction	0.1	0.0
E 100,000 × g supernatant fraction	0.2	0.2
F D + E	10.6	4.4

homogenate and in the combined microsomal and 100,000 × g supernatant fractions. Separate fractions were incapable of metabolizing CPR and NBA to any significant extent. It seems that the enzymes are located in the microsomal fraction of the liver.

Kinetics of liver enzyme activity.

In fig. 4 and 5 the Lineweaver-Burk plot for CPR and NBA is shown. The Michaelis constant was 0.77×10^{-4} M for CPR and 0.67×10^{-3} M for NBA. The maximal velocities were 0.29 and 0.08 μmol per gram tissue wet weight per hour, respectively. The magnitudes of the Michaelis constants seem to indicate that both CPR and NBA are "real" substrates for foetal enzymes.

Temperature and pH optimi for enzyme activities.

Optimal temperatures for both enzymes were in the region of 40 to 45°. Both enzymes have a rather broad pH optimum, the maximal point of which is about 7.6–7.8.

Cytochrome P-450.

We tried to determine cytochrome P-450 in two large foetuses (280 and 500 grams), in the tissues of which CPR and NBA metabolizing activity was detected. With the method and equipment used we could not identify the presence of cytochrome P-450 in liver microsomes. However, the bubbling of tissue preparation with carbon monoxide before the experiment abolished its drug-metabolizing activity.

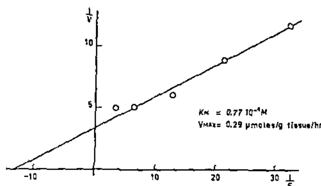


Fig. 4. Effect of substrate concentration on the metabolism of chlorpromazine. Cofactor mixture is described in Material and Methods. The amount of substrate varied from 0.016 to 0.156 μmol, and the 12,000 × g supernatant was used as an enzyme source. Incubation time was one hour.

Discussion

In our study with human foetal tissues we were able to detect low, but significant CPR metabolizing activity in the liver, intestine and kidney and also NBA metabolizing activity in the liver and intestine. As we reported earlier (PELKONEN *et al.* 1969), hexobarbitone and meperidine were metabolized in some trials, but their activities were not consistent. Experiments

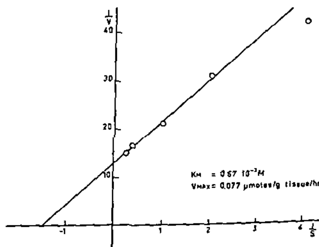


Fig. 5. Effect of substrate concentration on the metabolism *of p-nitrobenzoic* acid. Cofactor mixture is described *in Material and Methods.* The amount of substrate varied from *0.25 to 5.0* μmol, and the 12,000 × g supernatant was used in determinations. Incubation time was one hour.

with *p*-nitrophenol were unsuccesful, contrary to some previous results (DUTTON 1963).

We were also unable to detect cytochrome P-450 in the foetal liver microsomal fraction, although it has been found in the adult human liver (ALVARES *et al.* 1969) and in the human full-term placenta (MEIGS & RYAN 1968). However, the observed metabolism of CPR and NBA presupposes such a low concentration of cytochrome P-450 that it would be difficult to reveal this with the method and equipment used.

The data on drug metabolism in foetal and newborn life have been collected by DONE in his review articles (DONE 1964, 1966 & 1967). From the studies which have been made with newborn and foetal animals some conclusions can be drawn: 1) oxidative and reductive detoxification processes have been found uniformly to be deficient, and 2) there is less uniformity among studies of conjugative processes, the findings of which vary somewhat depending on the species, substrate or the type of tissue preparation used. Most processes are, however, deficient.

According to the above mentioned data there are few studies on the drug-metabolizing activity of the human foetus and newborn and these are mostly *in vivo* studies. Most of them have been planned to reveal the conjugative ability of the human foetus and newborn and up to the present only the acetylation of *p*-aminobenzoate has been found to be active (VEST & ROSSIER 1963). There is only scanty information about the oxidative and reductive ability of the human foetus and newborn and the results are negative. Aromatic hydroxylation of acetanilide *in vivo* (VEST & STREIFF 1959) and keto-reduction of cortisol *in vivo* (MIGEON 1959) are both deficient in the newborn human.

Recently many studies on drug metabolism in the human placenta have been published (JUCHAU *et al.* 1968a & b; JUCHAU 1969). Investigators were unable to detect various oxidative pathways, but they found that the placenta reduced the azo and nitro groups. WELCH *et al.* (1969) found that women who smoke cigarettes have high levels of 3,4-benzpyrene hydroxylase and aminoazo dye N-demethylase activity in the placenta, while non-smokers have little or no enzyme activity.

Looking at our results from a critical standpoint one must take into consideration the limits of assay methods and equipment in determining low activities and small concentrations. Our assays for CPR and NBA are more accurate than the assays for hexobarbitone, meperidine and *p*-nitrophenol. It is quite possible, that if more accurate methods were available, the metabolic activity for other drugs would also be found.

It has been shown in adult animals that CPR and NBA are metabolized in liver microsomes through the $NADPH_2$-dependent enzyme system in which cytochrome P-450 is the terminal oxidase (WILLIAMS 1961; GILLETTE 1963;

PARKE 1968; GILLETTE *et al.* 1968). For example, KUNTZMAN *et al.* (1966) and ACKERMANN & HEINRICH (1970) have shown that human liver also contains enzyme systems which catalyze different oxidative and reductive biotransformations. In many instances these enzyme systems were found to be qualitatively similar to those found in the rat. Since cytochrome P-450 has also been found in human liver microsomes (ALVARES *et al.* 1969) it is probable that oxidative drug metabolism in human liver is mediated through a similar enzyme system as in experimental animals. It seems probable that differences are qualitative in some cases but mostly quantitative in nature.

Our results show that the enzymes metabolizing CPR and NBA in the human foetus, are located in the liver microsomal fraction, require $NADPH_2$ or NADP and 100,000 × g supernatant fraction for full activity and are inhibited by carbon monoxide. The Michaelis constants were 0.77×10^{-4}M for CPR and 0.67×10^{-3}M for NBA. The kinetic constants for human foetus are of the same order of magnitude as for adult animals, for example the Michaelis constants were 1.8×10^{-4}M for CPR in the rat liver enzyme (RUBIN *et al.* 1964) and 0.68×10^{-3}M for NBA in the mouse liver enzyme (SASAME & GILLETTE 1969). The above mentioned characteristics of the foetal enzymes strongly suggest that they belong to the same class of enzymes as those detected in animal and human adults.

The maximal velocities for CPR and NBA metabolizing enzymes were 0.29 and 0.08 μmol per gram of tissue per hour. The values for rat liver enzymes were 6.7 (RUBIN *et al.* 1964) and 1.4 (KATO *et al.* 1969) respectively. Thus the activity of foetal enzymes is only about 5 % of the activity of adult rat liver enzymes. However, in many of the cases studied the activity of drug metabolizing enzymes in adult human liver is much smaller than in rat liver (KUNTZMAN *et al.* 1966).

According to FOUTS & HART (1965) there are two reasons for the deficiency in drug-metabolizing enzyme activity in the livers of foetal rabbits: 1) a lack of stimulus to the enzyme forming system since provision of a stimulator such as phenobarbital (phenemalum NFN) could cause increased drug metabolism – a state existing in late foetal life and early neonatal period and 2) some defect in the enzyme forming system which makes a response to a stimulator impossible – i. e. a condition existing throughout foetal life until the last two-three days before birth. In our studies a low basal activity was present in the first half of foetal life, and the characteristics of liver enzyme activity were comparable with those of adult enzymes. We also tried to induce these enzymes with phenobarbital given to mothers over a period of one-two weeks before the abortion but the experiments have so far been unsuccesful (unpublished experiments). It seems that the second alternative of FOUTS & HART (1965) is also more probable with regard to the human foetus, and that the defect possibly involves the regulation of the

enzyme forming system. KLINGER (1970) is of the opinion that the rapid rise in drug-metabolizing enzyme activity just after birth is due to the de-repression phenomenon, but we do not know the regulating signal. Moreover the question of some factor which inhibits induction during the foetal period still remains unanswered.

REFERENCES

Ackermann, E. & I. Heinrich: Die Aktivität der N- und O-demethylase in der Leber des Menschen. *Biochem. Pharmacol.* 1970, **19**, 327–342.

Alvares, A. P., G. Schilling, W. Levin, R. Kuntzman, L. Brand & L. C. Mark: Cytochromes P-450 and b5 in human liver microsomes. *Clin. Pharmacol. Ther.* 1969, **10**, 655–659.

Done, A. K.: Developmental pharmacology. *Clin. Pharmacol. Ther.* 1964, **5**, 432–479.

Done, A. K.: Perinatal pharmacology. *Ann. Rev. Pharmacol.* 1966, **6**, 189–208.

Done, A. K.: Neonatal and infant pharmacology. In: *Animal and clinical pharmacologic techniques in drug evaluation.* Vol. 2. Ed. P. E. Siegler and J. H. Moyer, Chicago 1967, pp. 130–140.

Dutton, G. J.: Comparison of glucuronide synthesis in developing mammalian and avian liver. *Ann. N. Y. Acad. Sci.* 1963, **111**, 259–272.

Fouts, J. R. & B. B. Brodie: The enzymatic reduction of chloramphenicol, *p*-nitrobenzoic acid and other aromatic nitro compounds in mammals. *J. Pharmacol. exp. Ther.* 1957, **119**, 197–206.

Fouts, J. R.: Metabolism of drugs by livers from fetal and newborn animals. In: *Perinatal pharmacology.* Ed.: C. D. May. Report of Fortyfirst Ross Conference on Pediatric Research, Columbus, 1962, Ross Laboratories, pp. 54–61.

Fouts, J. R. & L. G. Hart: Hepatic drug metabolism during the perinatal period. *Ann. N. Y. Acad. Sci.* 1965, **123**, 245–251.

Gillette, J. R.: Metabolism of drugs and other foreign compounds by enzymatic mechanisms. *Proc. Drug Res.* 1963, **6**, 11–73.

Gilette, J. R., J. J. Kamm & H. A. Sasame: Mechanism of *p*-nitro-benzoate reduction in liver: The Possible role of cytochrome P-450 in liver microsomes. *Molec. Pharmacol.* 1968, **4**, 541–548

Juchau, M. R., J. Krasner & S. J. Yaffe: Studies on reduction of azo-linkages in human placental homogenates. *Biochem. Pharmacol.* 1968a, **17**, 1969–1976.

Juchau, M. R., K. R. Niswander & S. J. Yaffe: Drug metabolizing systems in homogenates of human immature placentas. *Amer. J. Obstet. Gynec.* 1968b, **100**, 348–356.

Juchau, M. R.: Studies on the reduction of aromatic nitro groups in human and rodent placental homogenates. *J. Pharmacol. exp. Ther.* 1969, **165**, 1–8.

Kato, R., P. Vassanelli, G. Frontino & E. Chiesara: Variation in the activity of liver microsomal drugmetabolizing enzymes in relation to the age. *Biochem. Pharmacol.* 1964, **13**, 1037–1051.

Kato, R., T. Oshima & A. Takanaka: Studies on the mechanism of nitro reduction by rat liver. *Molec. Pharmacol.* 1969, **5**, 487–498.

Klinger, W.: Age dependence of enzyme induction by drugs. *Scand. J. clin. Lab. Invest.* 1970, **25**, suppl. 113, 9.

Kuntzman, R., L. C. Mark, L. Brand, M. Jacobsen, W. Levin & A. H. Conney: Metabolism of drugs and carcinogens by human liver enzymes. *J. Pharmacol. exp. Ther.* 1966, 152, 151–156.

Meigs, R. A. & K. J. Ryan: *Cytochrome P-450 and steroid biosynthesis in the placenta. Biochem. Biophys. Acta* 1968, 165, 476–482.

Migeon, C. J.: Cortisol production and metabolism in the neonate. *J. Pediat.* 1959, 55, 280–295.

Parke, D. V.: Factors which affect the metabolism of drugs. In: *Recent advances in pharmacology*. Ed. J. M. Robson and R. S. Stacey, London 1968, pp. 75–98.

Pelkonen, O., M. Vorne & N. T. Kärki: Drug-metabolizing activity in the liver, intestine and kidney of human foetus. *Acta Phys. Scand.* 1969, suppl. 330, 69.

Rubin, A., T. R. Tephly & G. J. Mannering: Kinetics of drug metabolism by hepatic microsomes. *Biochem. Pharmacol.* 1964, 13, 1007–1016.

Salzman, N. P. & B. B. Brodie: Physiological disposition and fate of chlorpromazine and a method for its estimation in biological material. *J. Pharmacol. exp. Ther.* 1956, 118, 46–56.

Sasame, H. A. & J. R. Gillette: Studies on the relationship between the effects of various substances on absorption spectrum of cytochrome P-450 and the reduction of *p*-nitrobenzoate by mouse liver microsomes. *Molec Pharmacol.* 1969, 5, 123–130.

Schenkman, J. B., H. Remmer & R. W. Estabrook: Spectral studies of drug interactions with hepatic microsomal cytochrome. *Mol. Pharmacol.* 1967, 3, 113–123.

Welch, R. M., Y. E. Harrison, B. W. Gommi, P J. Poppers, M. Finster & A. H. Conney: *Stimulatory effect of cigarette smoking on the hydroxylation of* 3,4-benzpyrene and the N-demethylation of 3-methyl-4-monomethylamino-azobenzene by enzymes in human placenta. *Clin. Pharmacol. Ther.* 1969, 10, 100–109.

Vest, M. F. & R. Rossier: Detoxication in the newborn: The ability of the newborn infant to form conjugates with glucuronics acid, glycine, acetate and glutatione. *Ann. N. Y. Acad. Sci.* 1963, 111, 183–197.

Vest, M. F. & R. R. Streiff: Studies on glucuronide formation in newborn infants and older children. *Amer. J. Dis. Child.* 1959, 98, 688–693.

Williams, R. T.: *Detoxication mechanisms*. London 1959.

Acta pharmacol. et toxicol. 1971, 29, 295–302.

From the Department of Pharmacology, University of Umeå,
Umeå, Sweden

Determination of Thioxanthenes in Plasma at Therapeutic Concentrations

By

T. Mjörndal and L. Oreland

(Received July 7, 1970)

Abstract: A method for measuring thioxanthenes at therapeutic levels in plasma, based on the measurement of the fluorescence of the oxidized drugs, has been developed. The method permits estimation of about 3 ng of the drug. The disappearance of the drug from the plasma was followed after intravenous administration to rabbits and oral administration of a therapeutic dose to human volunteers. N-demethylthiothixene is identified as a metabolite of thiothixene in man.

Key-words: Thioxanthenes – thiothixene – N-demethylthiothixene – determination of thioxanthenes.

Due to lack of methods for the measurement of small amounts of thioxanthenes there is no information on the blood plasma values of human subjects receiving the usual therapeutic doses of these drugs. The most sensitive method hitherto described for the determination of phenothiazines and related compounds is the fluorescence method of MELLINGER & KEELER (1964). A simplified version of this method has been published by PACHA (1969). In this communication we describe a modification of the method which increases the sensitivity for thioxanthenes sufficiently to allow of the estimation of plasma concentrations after therapeutic doses of these drugs. The method has been used in the study of the blood plasma values of a new potent thioxanthene, thiothixene, after intravenous administration to rabbits and oral administration in the usual therapeutic doses to man.

Materials and Methods

Thiothixene (navane®) was obtained from Chas Pfizer & Co. Inc., New York. Two possible metabolites of thiothixene, N-demethylthiothixene and thiothixene sulfoxide were obtained from Dr. H. Lahon, Brussels. Chlorprotixene-HCL, clopenthixol-2-HCl, and flupenthixol-2-HCl were supplied by H. Lundbeck & Co. AS, Copenhagen. n-Hep-

tane, "Spectranalyzed" and isoamyl alcohol, reagent grade, were obtained from Fischer Scientific Comp., Fair Lawn, New Jersey. Hydrogen peroxide was a product of Merck A. G., Darmstadt. All the other reagents used were of the highest grade commercially available.

0.28 mg/kg of thiothixene was administered intravenously to 3 adult rabbits. Blood samples from the rabbits were drawn from the central ear vein at regular intervals as described by RODRIQUEZ-ERDMANN (1959). The blood samples were collected into heparinized tubes, protected from unnecessary exposure to light, and kept in the refrigerator until analysis.

20 mg of thiothixene was given *per os* as a single dose to 5 healthy male students. Venous blood samples were taken at 0, 1.5, 3, 6, 9, 12, 18 and 26 hours after the administration of the drug. The blood was drawn into 10 ml vacuum tubes containing anticoagulant (vacutainer®) and kept in the refrigerator until analysis.

Determination of thiothixene in plasma.

2 ml heparin-treated plasma was alkalinized with 0.5 ml 10 N-NaOH and extracted with 10 ml of n-heptane in a 25 ml glass-stoppered tube by shaking on an automatic shaker for 15 min. After centrifugation for 5 min. at 5000 × g, 9 ml of the heptane phase was transferred into another glass-stoppered tube using a pipette rinsed with isoamyl alcohol just before use. The drug was then extracted into 1.5 ml of 0.1 M-H_2SO_4 by shaking and centrifugation as described above.

To 1.0 ml of the aqueous phase was added 0.5 ml of a 2 M acetate buffer pH 5.5. The pH dependence of the fluorescence is shown in fig. 1. Oxidation was then performed by addition of 0.1 ml of a 0.1 per cent solution of potassium permanganate. After 5 min. the excess of potassium permanganate was reduced by adding 0.1 ml of a 0.1 per cent solution of hydrogen peroxide. The fluorescence of the oxidized drug was determined at 310 nm activating and 440 nm fluorescent wavelengths (all wavelengths given are uncorrected instrumental values) with an Aminco-Bowman spectrofluorometer coupled to a Hewlett-Packard Moseley X-Y recorder. The fluorescent readings were then plotted against those obtained from known amounts of the drug added to human plasma.

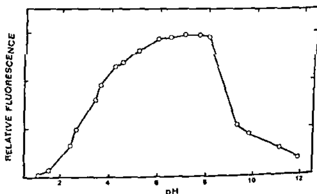


Fig. 1. The effect of various pH's on the fluorescence of thiothixene. 2 M acetate buffers with various pH's were used, and the drug was oxidized as described under Methods.

Thin-layer chromatography.

Thin-layer chromatography of alkaline heptane extracts from human plasma was performed on glass plates (20 × 20 cm) coated with a 250 μ layer of Silica Gel F_{254} (Merck). The plates were developed in the following solvent systems: 1) diethyl ether: diethylamine, 9 : 1, 2) acetone : n-heptane : diethylamine, 6 : 4 : 1 and 3) benzene : dimethylformamide : diethylamine, 8 : 1 : 1. These solvent systems gave good separations of thiothixene and the two thiothixene derivatives (table 2). The spots were visualized under UV-light and by spraying with concentrated H_2SO_4 (LAUFFER *et al.* 1969).

Results

Recovery.

In order to determine the recovery of thiothixene in the extraction procedure, known amounts of thiothixene were added to human plasma or water and carried through the entire procedure. The values obtained were then compared with those for the same amounts of the drug determined directly. The recoveries varied from about 70 to 100 per cent (table 1). The lower values

Table 1.

Recovery of thiothixene added to plasma.

Drug added ng/ml plasma	Recovery of thiothixene per cent	Recovery of N-demethyl-thiothixene per cent
1.5	65	–*
1.5	71	–
1.5	71	
5	74	30
5	90	33
5	82	
12.5	75	30
12.5	75	35
12.5	75	
25	89	43
25	99	47
25	99	
125	91	47
125	97	41
125	97	
250	100	43
250	99	41
250	99	

* not measurable.

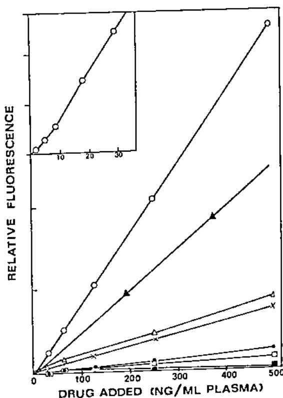


Fig. 2. The fluorescence of various psychopharmacological agents measured under optimal conditions for thiothixene.
○—○—○, thiothixene; △—△—△, clorprothixene; ×—×—×, clopenthixol, □—□—□, flupenthixol; ▲—▲—▲, thioridazine (Sandoz); ●—●—●, desipramine (Geigy); ■—■—■, dixyrazin (UCB 3412), levomepromazin, (levomepromazinum INN) (Leo), haloperidol (Leo) and chlorpromazine (Leo). For experimental details, see Results.

were obtained when small amounts of the drug were estimated, presumably because a proportionally larger amount of the drug was adsorbed to the glass-ware. No differences in the recoveries were observed when thiothixene was extracted from water instead of plasma.

Sensitivity.

The sensitivity of the method allowed the estimation of less than 1 ng when thiothixene was estimated without extraction. When thiothixene was extracted from plasma or water about 3 ng could be estimated (fig. 2). The intensity of fluorescence was linearly related to the concentration of drug up to 500 ng/ml plasma.

Specificity.

Since combinations of drugs are often used in psychiatric treatment the fluorescence of a number of psychopharmacological agents carried through the procedure was studied. In fig. 2 it is shown that the fluorescence of these is slight with the exception of thioridazine. The method is not restricted to the determination of thiothixene. By changing the pH to about 6 and the activating and fluorescent wavelengths to 390 and 450 nm, respectively, about the same sensitivity as for thiothixene was thus obtained for the other thioxanthenes tested, namely *chlorprotixene, clopenthixol and flupenthixol.*

Of particular interest was the influence of potential metabolites. Only the N-demethylated compound has hitherto been identified as a metabolite of thiothixene although several spots appear in chromatograms of organic solvent extracts of livers from rats treated with thiothixene (HOBBS 1968). N-demethylthiothixene showed the same fluorescence characteristics as the parent drug and a significant amount of the compound (table 1), was extracted into the heptane phase. The sulphoxide, a metabolite found in man after the administration of some other thioxanthenes (ALLGÉN *et al.* 1960; KHAN 1969) does not significantly interfere with the method.

Plasma levels after single doses of thiothixene.

When 0.28 mg/kg of thiothixene was administered intravenously to 3 adult rabbits the plasma levels of the drug decreased rapidly during the first hour and then decreased slowly. Most likely the rapid decrease during the first hour reflects a rapid redistribution of the drug while the subsequent slow decrease reflects its elimination (fig. 3).

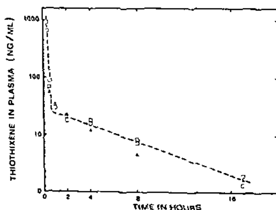


Fig. 3. Thiothixene in plasma after the intravenous administration of 0.28 . rabbits.

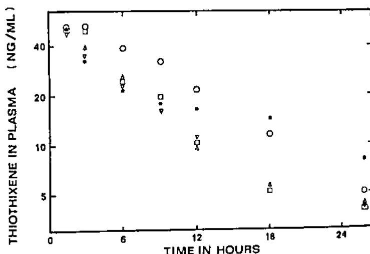


Fig. 4. Thiothixene in plasma after the oral administration of 20 mg to 5 male students.

In another experiment 20 mg of thiothixene was given *per os* to 5 healthy male students. This dose is often used in clinical practice (BISHOP *et al.* 1966; SIMEON *et al.* 1967). In fig. 4 the plasma levels of the drug are plotted against time.

As N-demethylthiothixene at least can interfere in the method (see above) chromatographic analyses of the heptane extracts of plasma after 2 and 4½

Table 2.

Rf-values in various solvent systems of thiothixene, thiothixene sulphoxide, N-demethylthiothixene and plasma extract 2 (t_1) and 4½ (t_2) hours after administration of thiothixene.

Solvent system	Thiothixene	Thiothixene sulphoxide	N-demethyl-thiothixene	Plasma extract* t_1	Plasma extract* t_2
Ether : diethylamine (9 : 1)	0.28	0.17	0.04	0.29	0.29
Acetone : n-heptane : diethylamine (6 : 4 : 1)	0.40	0.30	0.08	0.40	0.40 0.08
Benzene : dimethylformamide : diethylamine (8 : 1 : 1)	0.66	0.60	0.27	0.66	0.65 0.27

* For each spot 20 ml plasma was extracted.

hours were performed (table 2). In the chromatographic systems the heptane extract from human plasma taken 2 hours after the administration of the drug gave single spots with the same Rf-values as thiothixene. Chromatography of extracts from plasma after 4½ hours gave a major spot corresponding to thiothixene and in two of the systems one weak spot with the same Rf-value as N-demethylthiothixene. As judged from the intensity of this spot it constitutes less than 10 per cent of the material estimated.

Discussion

A good deal of effort has been directed towards developing methods for measuring major tranquilizers at therapeutic concentrations. A method for the determination of the phenothiazine thioridazine has recently been presented (PACHA 1969). This communication describes a modification of this method which permits the estimation of therapeutic concentrations of thioxanthenes.

By adjusting the pH and by changing the activating wavelengths the fluorescence readings were increased by about 50 per cent. More important, however, is the considerably higher sensitivity obtained by a reduction of the blank. Part of this reduction was achieved by extracting the drug into sulphuric acid instead of the strong acetate buffer used by RAGLAND *et al.* (1965) and PACHA (1969). Isoamyl alcohol, which is often used to reduce adsorption to rough surfaces (BRODIE 1947), was not necessary to get a good extraction of thiothixene and by excluding it, the blank values were further reduced. We also found that the amount of hydrogen peroxide could be considerably reduced as compared to that used by PACHA with a corresponding lowering of the blank.

After therapeutic doses of thiothixene had been given to 5 male students there was little variation in the plasma concentrations of the drug during the first few hours (fig. 4). Later on variations in the plasma levels were seen, reflecting the different elimination rates. Interference by metabolites may contribute to the deviations from the linear relationships between the logarithmic plasma values and time in the later part of the curves. By thin-layer chromatography of plasma drawn 4½ hours after the oral administration of 20 mg thiothixene it was shown that N-demethylthiothixene, a metabolite previously found in the rat (HOBBS 1968) is also formed in man. Thus after a few hours at least this metabolite may contribute to the fluorescent material found.

REFERENCES

Allgén, L.-G., B. Jónsson, B. Nauckhoff, M.-L. Andersen, I. Huus & I. Møller Nielsen On the elimination of chlorprotixene in rat and man. *Experentia* 1960, **16**, 325.

Bishop, M. P., T. E. Fulmer & D. M. Gallant: Thiothixene versus trifluoperazine in newly-admitted schizophrenic patients. *Curr. Ther. Res.* 1966, **8**, 509–514.

Brodie, B. B., S. Udenfriend & J. E. Baer: The estimation of basic organic compounds in biological material. 1. General principles. *J. Biol. Chem.* 1947, **168**, 299–309.

Hobbs, D. C.: Metabolism of thiothixene. J. Pharm. Sci. 1968, 57, 105–111.

Khan, A. R.: Some aspects of clopentixol metabolism in rats and humans. *Acta pharmacol. et toxicol. 1969, 27, 202–212.*

Lauffer, S., E. Schmid & F. Weist: Dünnschichtchromatographische Trennung und *spectrophoto-fluometrischer Nachweis psychotroper Pharmaka. Arzneimittelforsch.* 1969, **19**, 1965–1971.

Mellinger, T. J. & C. E. Keeler: *Factors influencing spectrofluorometry of phenothiazine drugs. Anal. Chem.* 1964, **36**, 1840–1847.

Pacha, W. L.: A Method for the fluorimetric determination of thioridazine (mellaril®) or mesoridazine (lidanil® in plasma. *Experientia* 1969, **25**, 103–104.

Ragland, J. B., V. J. Kinross-Wright & R. S. Ragland: Determination of phenothiazines in biological samples. *Anal. Biochem.* 1965, **12**, 60–69.

Rodriguez-Erdmann, F.: Über eine einfache Methode zur wiederholten Blutgewinnung beim Kaninchen. *Pflügers Arch.* 1959, **269**, 306–307.

Simeon, J., A. Keskiner, D. Ponce, T. Ztil & M. Fink: Clinical trial of navane (thiothixene) in schizophrenia. *Curr. Ther. Res.* 1967, **9**, 10–16.

Acta pharmacol. et toxicol. 1971, **29**, 303–313.

From the Department of Pharmacology, University of Göteborg, Göteborg, Sweden

Quantitative Estimation of 5-Hydroxy-3-Indole Acetic Acid and 5-Hydroxytryptophan in the Brain Following Isolation by Means of a Strong Cation Exchange Column

By

Margit Lindqvist

(Received August 12, 1970)

Abstract. Brain tissue is extracted with perchloric acid. The pH of the extract is adjusted to 2.5 and passed through a Dowex 50 W–X4 column. 5-Hydroxy-3-indole acetic acid is eluted with 60 % aqueous methanol, and then 5-hydroxytryptophan is eluted with 0.1 M phosphate buffer, pH 6.5. Fluorometric assays are performed according to the method for 5-hydroxytryptamine described by ANDÉN & MAGNUSSON (1967). The results are satisfactory with regard to reproducibility, recovery, specificity, and sensitivity. If the above procedure is combined with that published by ATACK & MAGNUSSON (1970), then 7 different compounds can be estimated after a single column procedure.

Key-words: 5-Hydroxy-3-indole acetic acid – 5-hydroxytryptophan – Dowex 50 W–X4.

Solvent extractions for the isolation of 5-hydroxy-3-indole acetic acid (5-HIAA) have been used by ROOS (1962) and GIACALONE & VALZELLI (1966). CONTRACTOR (1966) developed a method for the determination of 5-HIAA in urine using a Sephadex G-10 column. This method has been modified by JONSSON & LEWANDER (1970) for the separation of 5-HIAA and 5-hydroxytryptamine (5-HT) in the brain and cerebrospinal fluid.

WIEGAND & SCHERFLING (1962) have separated 5-hydroxytryptophan (5-HTP) and 5-HT on a Dowex 50W-X4 column following solvent extraction according to the method of SHORE & OLIN (1958).

In the present paper the isolation of 5-HIAA and 5-HTP on a Dowex 50W-X4 column and their subsequent determination are described. ATACK & MAGNUSSON (1970) have shown that noradrenaline and adrenaline, dopamine, 5-HT, and histamine can be isolated from the same column. Th[illegible] procedures can be combined.

Methods

One rat brain (1.5–1.7 g) is homogenized (Ultra-Turrax) in 10 ml ice-cold 0.4 N perchloric acid (PCA) containing 0.1 ml 2 % ascorbic acid and 0.2 ml 10 % EDTA (disodium ethylenediamine tetraacetate). The homogenate is centrifuged at about 14000 × g for 10 min. at 0°. Any remaining particles on the surface of the supernatant are removed by filtration. The pH is adjusted to 2.5 with 5 N potassium carbonate, the sample is cooled to 0° and the precipitate is spun down.

For the ion exchange procedure the apparatus described by BERTLER *et al.* (1958) is used.

Dowex 50 W-X4 columns, 200 to 400 mesh, 4.2 × 70 mm are pretreated as follows.
1. 20 ml 2 N-NaOH containing 1 % EDTA.
2. Water in sufficient amount to remove the NaOH.
3. 20 ml 2 N-HCl.
4. Water to remove the acid.
5. 20 ml 0.1 M phosphate buffer, pH 6.5 containing 0.1 % EDTA.
6. 5 ml water.

The length of the column is then 60 mm.

The sample is then passed through the column at a rate not higher than 0.4 ml/min. 5-HIAA is eluted by 10 ml 60 % aqueous methanol, rate 0.4 ml/min. The apparatus is carefully washed with water to remove any methanol present and then 2 × 5 ml water are passed through the column. A second eluate of 10 ml 0.1 M phosphate buffer, pH 6.5 containing 0.1 % EDTA is collected and this contains 5-HTP.

Immediately after the elution, 5-HIAA and 5-HTP are determined fluorometrically according to the method for 5-HT described by ANDÉN & MAGNUSSON (1967). An aliquot of 0.6 ml is put into a silica test tube together with 0.1 ml 1 % ascorbic acid and 0.1 ml 0.025 % potassium ferricyanide. Then 0.8 ml 6 N-HCl is added and the sample is irradiated for 10 min. under a UV lamp. A standard, a reagent blank and an internal standard are run in parallel with the sample. A tissue blank is treated in the same way except that the ascorbic acid is added after the UV irradiation. The fluorescence is read against a standard set to 50 in an Aminco-Bowman spectrophotofluorometer at the activating and fluorescence peaks 295/545 mμ (uncorrected instrumental values). A UV filter is placed in front of the photocell for elimination of the second order light scatter peak at 590 mμ.

In some experiments 5-HIAA-carboxyl-^{14}C (6.44 mci/mmole) has been used. The purity was checked by paper chromatography (20 % KCl, ascending) and subsequent scanning of the paper for radioactivity. In the experiments radioactivity was measured by liquid scintillation (Packard Tri Carb) using Insta-Gel as the emulsifying scintillation mixture.

Results and Comments

Extraction procedure. Recovery.

To check the extraction of 5-HIAA from brain tissue ^{14}C-labelled 5-HIAA was added to the homogenates. Single rat brains were extracted 5 times with 10 ml 0.4 N-PCA (6 ml/g brain) containing 0.1 ml 2 % ascorbic acid and 0.2 ml 10 % EDTA. In the first extraction only about 50 % of the added 5-HIAA was recovered. The total amount extractable was 80 %, indicating that 5-HIAA may be bound to the solid residue of brain tissue (table 1).

Table 1.

Recovery of ^{14}C-labelled 5-hydroxy-3-indole acetic acid (30 ng added to the homogenate) after 5 successive extractions of a single rat brain. The amount of radioactivity is expressed as per cent of the added amount.

Extraction no.:	I	II	III	IV	V	Total
Brain 1	47	22	6	2	0	77
Brain 2	50	21	6	2	1	80

Note: Brain 1 weighed 1.59 g and would thus presumably contain 1.19 ml water (*assuming a water content of 75 %). The total volume of water in the homogenate was* $10 + 0.1 + 0.2 + 1.19 = 11.49$ ml. The volume of the supernatant obtained after extraction I was 9.9 ml or 86 % of the total volume of water in the homogenate. The recovery from a single extraction, corrected for this fluid loss, is 54 % for brain 1, and 58 % for brain 2.

Following the addition of larger amounts of unlabelled 5-HIAA somewhat higher recoveries were obtained (table 3).

Table 2 shows the recoveries of unlabelled 5-HTP added to homogenates of 3 pooled rat brains in 15 ml 0.4 N-PCA (3 ml/g brain), 0.15 ml 2 % ascorbic acid and 0.30 ml 10 % EDTA. Two extractions were made. In the first extraction about 60 % and in the second 18 % of the added amount was found.

Homogenates of 2 rat brains were each divided into two equal parts. To one part a known amount (0.5 to 10 μg) of 5-HIAA and/or 5-HTP was added. The two parts were run in parallel through the procedure. After sub-

Table 2.

The content of 5-hydroxytryptophan in the supernatant after 2 successive extractions of 3 pooled rat brains. The figures are given as per cent of the total amount of 5-HTP (10 μg) added to the homogenate.

Extraction no.:	I	II	Total
Sample 1	61	18	79
Sample 2	61	18	79

Note: The brain tissue weighed 5.43 g and would thus presumably contain 4.07 ml water (assuming a water content of 75 %). The total volume of water in the homogenate was $15 + 0.15 + 0.30 + 4.07 = 19.52$ ml. The volume of the supernatant obtained after extraction I was 14 ml or 72 % of the total volume of water in the homogenate. The recovery from a single extraction, corrected for this fluid loss, is 85 % for sample 1, 82 % for sample 2.

Table 3.

Recoveries in per cent of 5-hydroxy-3-indole acetic acid added to homogenates of rat brains. Figures in brackets indicate number of determinations.

Amount of 5-HIAA added (μg)	0.5	1	10
Two extractions (3 + 2.5 ml PCA per g brain)		65 (3) ± 7.1 (S. E. M.) ± 12.3 (S. D.)	77 (2) ± 3.5 (S. E. M.) ± 5.0 (S. D.)
Single extraction *(6 ml PCA per g brain)*	67 (5) ± 5.6 (S. E. M.) ± 12.4 (S. D.)	71 (12) ± 2.4 (S. E. M.) ± 8.3 (S. D.)	83 (6)* ± 4.2 (S. E. M.) ± 10.3 (S. D.)

* In these experiments only 7.5 μg 5-HIAA was added.

tracting the endogenous content, the recoveries were calculated as per cent of the added amounts. The recoveries of 5-HIAA after two extractions with 3 + 2.5 ml PCA per g brain or after a single extraction (corrected for 75 % water content in the brain) with 6 ml PCA per g brain were 65 to 77 % and 67 to 83 %, respectively (table 3).

The recovery of 5-HTP was 86 to 93 % after two extractions and 95 to 97 % after a single extraction (table 4).

The single extraction procedure was used in the following experiments described in this paper.

Extracts were usually kept overnight at −20° before the ion exchange procedure. The stability was checked at pH 2.5 and also at pH 6 by measur-

Table 4.

Recoveries, in per cent, of 5-hydroxytryptophan added to homogenates of rat brains. Figures in brackets indicate number of determinations.

Amount of 5-HTP added (μg)	1	2	10
Two extractions (3 + 2 ml PCA per g brain)	93 (2) ± 0.5 (S. E. M.) ± 0.7 (S. D.)	88 (1)	86 (4) ± 3.5 (S. E. M.) ± 7.0 (S. D.)
Single extraction (6 ml PCA per g brain)		97 (9)* ± 2.2 (S. E. M.) ± 6.7 (S. D.)	95 (3) ± 6.9 (S. E. M.) ± 11.9 (S. D.)

* In these experiments 7.5 μg 5-HTP was added.

ing 5-HIAA and 5-HTP immediately, on the next day and after 3 and 7 days, with portions of the same extract. A continuous loss occurred and on day 3 it was 5 % for both 5-HIAA and 5-HTP. On day 7 a loss of 8 to 15 % was found. No significant pH dependence was detectable.

Column procedure.

The separation of 5-HIAA and 5-HTP is shown in fig. 1. To a model extract consisting of 1.5 ml 0.9 % saline, 1 ml 4 N-PCA, 0.1 ml 2 % ascorbic acid, 0.2 ml 10 % EDTA and 7.5 ml water 10 μg 5-HIAA and 10 μg 5-HTP were added. The sample was run through the procedure, and effluent, eluates and water were collected in 1 ml fractions. One ml 6 N-HCl was added to each fraction which was then read in the spectrophotofluorometer at 295/545 mμ.

5-HIAA showed a low affinity to the cation exchange column. In view of slight (or absent) cationic properties of this molecule under the present conditions it was, in fact, surprising that it had any affinity at all to a cation exchange column. The break-through capacity of the column is illustrated in fig. 2. These findings suggested that the passage of more than 10 ml extract through the column would result in significant loss of 5-HIAA.

For the 5-HTP determination 10 g brain tissue extracted in 60 ml PCA could be passed through the column without any break-through.

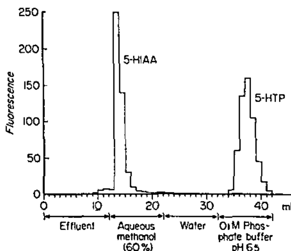


Fig. 1. Separation of 5-hydroxy-3-indole acetic acid (10 μg) and 5-hydroxytryptophan (10 μg) on a strong cation exchange column (Dowex 50W-X4, 4.2 mm (I. D.) × 60 mm, Na^+-form) Effluent, water and eluates were collected in 1 ml fractions. After the addition of 1 ml 6 N-HCl, the fractions were read in a spectrophotofluorometer at 295/545 mμ.

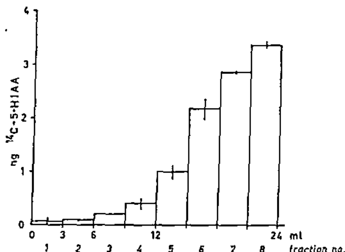


Fig. 2. The break-through capacity of a Dowex 50W-X4 column (4.2 mm (I.D) × 60 mm, Na^+-form). Brain extract, containing 30 ng ^{14}C-labelled 5-hydroxy-3-indole acetic acid was passed through the column. The effluent was collected in 3 ml fractions. Radioactivity was measured by liquid scintillation. Vertical bars indicate range of two determinations.

The methanol elution of 5-HIAA was done immediately after the extract had passed onto the column. Elution could also be performed with water, although a larger volume was then required. After the elution of 5-HIAA the apparatus and column must be washed carefully with water before the elution of 5-HTP. Methanol present in the buffer eluate increased the fluorescence of 5-HTP.

After the elution of 5-HTP the column can be washed with water and elution of noradrenaline and adrenaline, dopamine, 5-HT, and histamine can be performed by a technique developed in this laboratory by ATACK & MAGNUSSON (1970). Thus the combination of the two procedures results in the separation of 6 different compounds after a single column procedure.

The present method has also been used for the determination of 3,4-dihydroxyphenylalanine (DOPA) in human plasma. Nine ml blood are collected in a plastic tube containing 1 ml saline with 1 % EDTA. After centrifugation the plasma is removed by means of a pipette. 1/10 volume of 4 N-PCA is added and the proteins are spun down. Two ml of the extract are made up to 10 ml with water and 0.1 ml 2 % ascorbic acid and 0.2 ml 10 % EDTA are added. After adjustment of the pH to 2.5 and centrifugation the extract is passed through the column. After washing with 20 ml water, 20 ml 60 % aqueous methanol and another 20 ml water, the elution is performed with 5 ml 0.1 M phosphate buffer, pH 6.5 without EDTA. The eluate is collected in a flask standing in ice-water. Immediately after the elution the sample is

oxidized according to the method for noradrenaline described by BERTLER *et al.* (1958), except that the oxidation time is reduced to 3 min. The fluorescence is read at 360/470 mμ in the spectrophotofluorometer.

Reproducibility.

An extract from 7 pooled rat brains was divided into 7 equal samples which were run simultaneously. The amount of 5-HIAA per sample was found to be 0.30 μg ± 0.06 (mean ± S. D.). The coefficient of variation was thus 20 %. This seems to be a reasonable value considering the fact that the reading of the sample was 2 to 3 times that of the tissue blank.

Seven μg 5-HTP were added to a homogenate from 7 pooled rat brains. The extract was divided into 7 equal samples which were run simultaneously. The amount of 5-HTP per sample was 0.78 μg ± 0.05 (mean ± S. D.), resulting in a coefficient of variation of 6.4 %.

Table 5.

The fluorescence and the behaviour on a strong cation exchange column of some 5-OH and 5-OCH_3-indole compounds.

	Relative fluorescence Intensity	Appearing in	
		Eluate 1	Eluate 2
5-Hydroxytryptophan	60	−	+
α-Methyl-5-hydroxytryptophan	60	−	+
5-Hydroxytryptamine	66	−	−
α-Methyl-5-hydroxytryptamine	65	−	−
N-Acetyl-5-hydroxytryptamine	20	+	−
N, N-Dimethyl-5-hydroxytryptamine (Bufotenin)	68	−	−
5-Methoxytryptamine	55	−	−
N-Acetyl-5-methoxytryptamine (Melatonin)	17	+	−
5-Hydroxy-3-indole acetic acid	50*	+	−
5-Hydroxytryptophol	26	+	−

* Set to 50.

The concentration of the indoles was 1 μg/2 ml. The blanks were 0.5 to 1 unit. Activating and fluorescence peaks were 295/545 mμ.

The compounds were put on columns (Dowex 50W, X4, Na^+-form) and eluted with 10 ml 60 % aqueous methanol (Eluate 1) and then with 10 ml 0.1 M phosphate pH 6.5, containing 0.1 % EDTA (Eluate 2).

Specificity.

A complete separation of 5-HIAA from 5-HTP on a Dowex 50W, X4 column is obtained (fig. 1). The behaviour on the column of some other 5-OH and 5-OCH_3-indoles was also investigated. Of the compounds tested, N-acetyl-5-hydroxytryptamine, N-acetyl-5-methoxytryptamine (melatonin) and 5-hydroxytryptophol were found in the methanol fraction together with 5-HIAA. α-Methyl-5-hydroxytryptophan together with 5-HTP were found in the buffer fraction. The fluorescence spectra of all these 5-hydroxy and 5-methoxy indoles were identical (cf. ANDÉN & MAGNUSSON 1967) (table 5).

Melatonin and N-acetyl-5-hydroxytryptamine occur almost exclusively in the pineal gland and in relatively small amounts as compared to 5-HIAA in the whole brain (QUAI 1964; MAICKEL & MILLER 1968). However, under conditions where 5-HIAA levels are much below normal these compounds can be of importance. Under such conditions it is advisable to remove the pineal gland before the extraction.

5-Hydroxytryptophol does not seem to occur in appreciable amounts in the brain (JONSSON & LEWANDER 1970).

Extracts, each from 2 rat brains, were divided into two equal parts. One was run using the present method and the other using the method of JONSSON & LEWANDER (1970) on Sephadex G-10 columns. The results were in reasonably good agreement which lends further support to the specificity (table 6).

As α-methyl-5-hydroxytryptophan does not normally occur in the brain there are no known endogenous indole compounds which can interfere with the 5-HTP determination.

Sensitivity.

5-HIAA. Using standard conditions with pure reagents a reading of twice the reagent blank corresponds to 6 ng 5-HIAA in the sample (100 ng/eluate).

Table 6.

Assay of 5-hydroxy-3-indole acetic acid. Comparison of the present method with that of JONSSON & LEWANDER (1970).

	Dowex 50W–X4	*Sephadex G-10*
Brain 1	0.20	0.21
Brain 2	0.21	0.24
Brain 3	0.13	0.15

Values are μg/g brain, uncorrected for a mean recovery of 72 % on the Dowex columns and of 61 % on the Sephadex columns. The difference between the recoveries is not statistically significant.

Table 7.

Assay of 5-hydroxytryptophan in brain. Actual readings are shown.

Eluate derived from	Standard 0.2 μg	Reagent blank	Sample	Internal standard	Tissue blank
2 g rat brain	50	0.5	6.0	50.5	5.0
2 g rat brain	50	0.5	4.0	50.0	4.5
5 g mouse brain	50	1.0	6.0	53.5	6.5
5 g mouse brain	50	1.0	5.5	55.0	5.5

Using brain extract a reading twice the tissue blank corresponds to 18 ng in the sample (300 ng/eluate) and to 0.23 μg/g brain.

The sensitivity of the present method is lower than for the method with Sephadex G-10 columns (JONSSON & LEWANDER 1970). However, the advantage is the isolation of 5-HIAA and 5-HTP, 5-HT and several other amines, using a single column procedure.

5-HTP. Using pure reagents a reading twice the reagent blank corresponds to the presence of 4 ng 5-HTP in the sample (67 ng/eluate). Using brain extracts a reading twice the tissue blank, corresponds to 18 ng in the sample (300 ng/eluate), or 0.22 μg/g brain.

Levels of 5-HIAA and 5-HTP in rat brain.

The normal level of 5-HIAA in brains of male Sprague-Dawley rats (body weight 180 to 220 g) was found to be 0.28 ± 0.03 μg/g (mean ± S. E. M.; S. D. = 0.08) in 10 determinations on single brains. The values are corrected for 68 % recovery.

Using Sephadex G-10 columns JONSSON & LEWANDER (1970) have reported a normal level of 5-HIAA of 0.28 ± 0.01 μg/g brain (mean ± S. E. M., n = 31) in male Sprague-Dawley rats weighing 180 to 210 g. Their values were uncorrected for a recovery of 91 %. Thus, our findings are in reasonably good agreement with those of JONSSON & LEWANDER and also with those of several other investigators (ASHCROFT *et al.* 1965; JUORIO & VOGT 1965; PLETSCHER *et al.* 1964; GIACALONE & VALZELLI 1966; cf. also table 6).

Table 7 shows the readings for assays of 5-HTP in rat and mouse brains. The readings of the samples are very similar to the readings of the tissue blanks. All the readings are less than 2 units over the tissue blank. Hence, the normal level of 5-HTP, calculated from 2 g rat brain or 5 g mouse brain, is probably less than 0.07 and 0.03 μg/g, respectively. WIEGAND & SCHERFLING (1962) reported the normal level of 5-HTP to be less than 0.1 μg/g rat brain.

GEY & PLETSCHER (1960) have shown that 5-HTP decarboxylation continues after the animals have been killed. Further investigations e. g. with rapid freezing in liquid air are desirable. However, experiments with mouse brains, dissected out and homogenized within 15 seconds, did not result in any measurable amounts of 5-HTP. It is doubtful if the usual procedure with liquid air will result in an appreciably more rapid interruption of the enzymatic processes.

If rats or mice are pretreated with an aromatic amino acid decarboxylase inhibitor, 5-HTP can be demonstrated in the brains (CARLSSON & LINDQVIST 1970).

Acknowledgements

This research was supported by the Swedish Medical Research Council (Grant No. B70-14X-155-06B). The technical assistance of Mrs. Ingrid Bergh and Mrs. Birgitta Nyström is gratefully acknowledged. For the experiments with the Sephadex procedure I am indebted to Dr. B. Werdinius and Miss A. Wallin.

REFERENCES

Andén, N.-E. & T. Magnusson: An improved method for the fluorimetric determination of 5-hydroxytryptamine in tissues. *Acta physiol. Scand.* 1967, **69**, 87–94.

Ashcroft, G. W., D. Eccleston & T. B. B. Crawford: 5-Hydroxyindole metabolism in rat brain. A study of intermediate metabolism using the technique of tryptophan loading – I. Methods. *J. Neurochem.* 1965, **12**, 483–492.

Atack, C. V. & T. Magnusson: Individual elution of noradrenaline (together with adrenaline), dopamine, 5-hydroxytryptamine and histamine from a single, strong cation exchange column, by means of mineral acid-organic solvent mixtures. *J. Pharm. Pharmacol.* 1970, **22**, 625–627.

Bertler, A., A. Carlsson & E. Rosengren: A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. Scand.* 1958, **44**, 273–292.

Carlsson, A. & M. Lindqvist: Accumulation of 5-hydroxytryptophan in mouse brain after decarboxylase inhibition. *J. Pharm. Pharmacol.* 1970, **22**, 726–727.

Contractor, S. F.: A rapid quantitative method for the estimation of 5-hydroxyindolacetic acid in human urine. *Biochem. Pharmacol.* 1966, **15**, 1701–1706.

Gey, K. F. & A. Pletscher: Post mortem increase of 5-hydroxytryptamine in rat brain after 5-hydroxytryptophan administration. *Experientia* 1960, **16**, 372–373.

Giacalone, E. & L. Valzelli: A method for the determination of 5-hydroxyindolyl-3-acetic acid in brain. *J. Neurochem.* 1966, **13**, 1265–1266.

Jonsson, J. & T. Lewander: A method for the simultaneous determination of 5-hydroxy-3-indole-acetic acid (5-HIAA) and 5-hydroxytryptamine (5-HT) in brain tissue and cerebrospinal fluid. *Acta physiol. Scand.* 1970, **78**, 43–51.

Juorio, A. V. & M. Vogt: The effect of prenylamine on the metabolism of catechol amines and 5-hydroxytryptamine in brain and adrenal medulla. *Brit. J. Pharmacol.* 1965, **24**, 566–573.

Maickel, R. P. & F. P. Miller: The fluorometric determination of indolealkylamines in brain and pineal gland. In: *Advances in pharmacology* 1968, 6A, 71–77. Editors: E. Costa & M. Sandler. New York, Academic Press.

Pletscher, A., W. P. Burkard & K. F. Gey: Effect of monoamine releasers and decarboxylase inhibitors on endogenous 5-hydroxy-indole derivatives in brain. *Biochem. Pharmacol.* 1964, **13**, 385–390.

Quay, W. B.: Circadian and estrous rhythms in pineal melatonin and 5-hydroxy indole-3-acetic acid. *Proc. Soc. exp. Biol. Med.* 1964, **115**, 710–713.

Roos, B.-E.: On the occurrence and distribution of 5-hydroxyindole-acetic acid in brain. *Life Sci.* 1962, **1**, 25–27.

Shore, P. A. & J. S. Olin: Identification and chemical assay of norepinephrine in brain and other tissues. *J. Pharmacol.* 1958, **122**, 295–300.

Wiegand, R. G. & E. Scherfling: Determination of 5-hydroxytryptophan and serotonin. *J. Neurochem.* 1962, **9**, 113–114.

Acta pharmacol. et toxicol. 1971, 29, 314–326.

From the Laboratory of Bacteriology, Institute of Pathology (Head: S. Fischer) and Surgical Unit K (Head: J. Lindenberg), Municipal Hospital of Frederiksberg, Copenhagen, Denmark

Doxycyclinum NFN: A Pharmacological and Bacteriological Investigation

By

M. Lykkegaard Nielsen, H. Laursen, J. Gauguin and P. Rosenkast

(Received September 2, 1970)

Abstract: In normal human subjects multiple dose trial with an initial dose of 400 mg doxycycline and subsequent doses of 200 mg every 24 hours gave average minimum serum concentrations of between 1 and 2 μg doxycycline/ml serum. The average urine concentrations varied between 100 and 200 μg doxycycline/ml urine, and 42 per cent of the drug administered was excreted in the urine in an active form. The average renal clearance of doxycycline determined over 96 hours was 28 ml/min. In a single dose trial with 200 mg doxycycline, an 8-hour renal clearance of doxycycline was found to be 13 per cent of the creatinine clearance, and the serum half-life period was found to be 11.6 hours. Microbiological assay was used for all determinations of doxycycline in the serum and urine. The *in vitro* sensitivity of 211 urinary tract pathogens to doxycycline and oxytetracycline was investigated in order to determine the minimum inhibitory concentration (MIC). The strains, which originated from patients with chronic urinary tract infections, were as a whole only slightly sensitive to the two tetracyclines. Compared with oxytetracycline, doxycycline, assessed at the MIC-level, showed a higher bacteriostatic effect *in vitro*, especially against gram-positive strains.

Key-words: Doxycycline – pharmacology – bacteriology.

The purpose of this investigation was to determine the serum concentration, the urine concentration, the half-life period, and the clearance of doxycyclinum (NFN). It was further attempted to determine the sensitivity of a wide range of commonly occurring urinary tract pathogens to doxycycline and oxytetracyclinum (NFN). Finally an attempt was made to compare the *in vitro* bacteriostatic activity of doxycycline with that of oxytetracycline.

Pharmacology. Doxycycline is alpha-6-desoxy-5-oxytetracycline, a semisynthetic tetracycline (fig. 1). Like other tetracyclines, doxycycline is assumed to be bacteriostatic and to act by inhibiting the protein synthesis of sensitive micro-organisms (WINKLER & WEIH 1967).

In human subjects a single dose of 200 mg results in the following serum

concentrations (Fabre *et al.* 1966; Dimmling 1967; Williamson 1967) after 3, 12, 24, 36, and 72 hours: 2.5–2.7, 1.5–2.2, 0.8–1.5, about 1.1, and about 0.1 μg/ml, respectively. The maximum concentration is reached between 2.25 and 9 hours after administration and ranges from 2.6 to 3.3 μg/ml serum.

Based on the excretion in the faeces during the first 24 hours, the intestinal absorption has been found to be more than 98 per cent (Fabre *et al.* 1966). The same absorption result is reached when the tissue concentration is regarded as being equal to the maximum serum concentration. The absorption is not affected by simultaneous intake of food, whereas it is inhibited by $AL(OH)_3$ (Migliardi & Schach von Wittenau 1966; Rosenblatt *et al.* 1966; Williamson 1967). The almost complete absorption is probably due to the fact that, unlike other tetracyclines, doxycycline does not form chelates with Ca- and Mg-ions (Migliardi & Schach von Wittenau 1966).

The elimination, completed in about 72 hours after a single dose, is due to urinary and faecal excretion, and to decomposition to products without antibacterial effects (Fabre *et al.* 1967).

The excretion in the urine amounts to about 20 per cent during the first 24 hours, 10 per cent during the next 24 hours, and 5 per cent during the following 24 hours (Fabre *et al.* 1966; Dimmling 1967). After the administration of 200 mg to normal subjects, the following concentrations have been found: 1–6 hours about 60 μg/ml and 6–12 hours, about 50 μg/ml urine (Dimmling 1967).

Owing to the slow elimination, accumulation is found to occur if treatment is continued (Migliardi & Schach von Wittenau 1966; Dimmling 1967). The half-life period is about 15 hours after a single dose of 200 mg (Fabre *et al.* 1966). If treatment is repeated once every 24 hours, the half-life period is raised to about 20 hours on the 4th day, after which the half-life period remains almost unchanged, at least until the 30th day (Fabre *et al.* 1966). No changes can be demonstrated in the mechanism with which the kidneys deals with doxycycline during prolonged administration.

Determination of clearance of the drug gives varying values, ranging from 15 to 31 ml/minute (Fabre *et al.* 1966; Rosenblatt *et al.* 1966).

Bacteriology. The antibacterial effect of doxycycline, determined by inhibition of bacterial growth *in vitro*, has been fairly widely studied, both for fresh isolates and for laboratory strains (Fabre *et al.* 1966; Dimmling 1967; Mon-

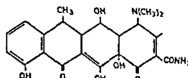


Fig. 1. Doxycyclinum. α-6-desoxy-5-oxytetracycline.

NIER & BOURSE 1967; REBER, personal communication 1967; WILLIAMSON 1967).

In the case of gram-positive bacteria, doxycycline is often found to have a greater bacteriostatic effect than other tetracyclines. This particularly applies to *staphylococcus aureus* and to *streptococcus faecalis*.

As to gram-negative bacteria, doxycycline usually proves to be just as bacteriostatic as other tetracyclines. *Mycobacterium tuberculosis* has been found to be more sensitive to doxycycline than to any other known tetracycline (WILLIAMSON 1967).

Material and Methods

The doxycycline concentration in the serum and urine was determined by the technique first described by VESTERDAL (1947), although in a slightly modified form, using 5 % blood agar as the upper layer in the plates. In order to obtain a uniform inoculum, the method of KIRSCHBAUM *et al.* (1962) was used.

The test strain used was *staphylococcus aureus* ATTC 6538 P.

For the calculation of standard curves, standard solutions of pure doxycycline sulphate were made: 30–15–7.5–3.78–1.89–0.95 and 0.48 μg/ml respectively. For the determination of the serum concentrations the drug was dissolved in human serum, while physiological saline was used for the determination of urine concentrations. Uniform experimental conditions were ensured by testing the standard solutions and the serum and urine with the unknown content of doxycycline on the same plate. The serum was examined partly undiluted and partly diluted 1 : 1. The urine was diluted with physiological saline, using two dilutions and most often 1 : 10 and 1 : 20. Zone diameters of the standard solutions were plotted against $\log_{10}$ of the concentrations, and the standard curve was drawn. In all cases the average values of 6 readings were used for the final determination of the doxycycline concentration in the blood and urine, obtained by interpolation along the standard curves.

The serum and urine concentrations were determined in 2 groups of 4 and 5 healthy young volunteers. The preparation used was vibramycin® in capsules of 100 mg.

Group I. A single dose of 200 mg was given with subsequent determinations of the concentrations during 30 hours. The excretion in the urine was determined over 8-hour period and the determinations of the serum and urine creatinine concentrations were performed at the same time. The clearance was calculated as a percentage of creatinine clearance by the standard formula $Cl = \frac{Cu \times D}{Cp}$, where Cl = clearance, Cu = concentration in urine, D = diuresis in ml/minute, and Cp = concentration in plasma. The excretion was regarded as an exponentially decreasing function (FABRE *et al.* 1966; ROSENBLATT *et al.* 1966), and the plasma half-life period was calculated by the standard formula $t_{1/2} = \frac{\log 2}{m}$, where $t_{1/2}$ = half-life in hours and m = slope of the regression curve.

Group II. At 8 a. m. and for 96 hours with intervals of 24 hours, an initial dose of 400 mg and subsequent doses of 200 mg were given. In 4 of the 5 volunteers the excretion in the urine was determined during the same period, the urine being collected for 4 days in 3 portions every 24 hours: at 8 a. m.–2 p. m., 2 p. m.–8 p. m., and 8 p. m.–8 a. m. A 96-hour clearance was calculated by the standard formula mentioned above.

In order to estimate the antibacterial activity of doxycycline and oxytetracycline, the minimum inhibitory concentration (MIC) of the two agents was measured. Freshly isolated pathogens from patients with urinary tract infections of chronic or recurrent type were used. All the strains of bacteria used were isolated and identified to species in accordance with the ordinary bacteriological methods. MIC-measurements were performed by growing pure cultures in broth with decreasing concentrations of the antibiotic in the following dilutions: 100-50-25-12.5-6.25-3.12-1.56 and 0.78 µg/ml. Inoculum: one drop of an 18-hour broth culture in 5 ml broth containing the antibiotic. Our criterion of difference in bacteriostatic activity of the two antibiotics was: a difference of the growth of at least two tubes of the dilutions (corresponding to a difference in the concentration of the antibiotics of at least four times).

Results

Table 1 shows the concentrations of doxycycline in the serum in the 4 volunteers in group I. The maximum concentration in the serum occurs from 2 to 4 hours after administration of the drug, and the values measured 24 hours after the intake were all more than 1 µg/ml.

Table 2 shows the excretion in the urine in the same test group. Within the first 8 hours nearly 7 per cent of the intake of doxycycline is excreted in an active form.

Table 3 and fig. 2 show the serum concentration of doxycycline in the 5 volunteers in group II. The values at 8 a. m. represent the minimum concentrations for each day. 2½ hours after the intake of the drug, the serum concentrations in 10 out of 20 measurements showed a lower level than 4½ hours after the intake, whereas in 5 cases the reverse was true. Taken as a whole, the individual results within each period show considerable variations.

Table 4 and fig. 3 represent the excretion in the urine in group II. An average of 417 mg was excreted in an active form (42 %) out of the total

Table 1.

Group I. Serum concentrations in 4 volunteers after intake of 200 mg doxycycline at 8 a. m. on the first day. µg doxycycline/ml serum.

Time	I. day					II. day			
	08	10	12	14	16	08	10	12	14
MS (59 kg)	0	3.0	3.0	2.7	2.0	1.2	0.8	0.6	0.2
BM (55 kg)	0	*	4.4	4.4	4.0	1.2	0.8	0.8	0.5
IS (57 kg)	0	2.8	3.8	2.7	3.8	1.2	0.8	0.5	0.6
ML (66 kg)	0	4.4	3.0	2.4	1.5	1.3	0.8	0.6	0.3
Average	0	3.4	3.6	3.1	2.8	1.2	0.8	0.6	0.4
Standard deviation		0.87	0.68	0.91	1.58	0.05	0.00	0.12	0.18

* = no determination made.

Table 2.

Group I. Concentration and excretion in the urine in 4 volunteers during the first 8 hours after the intake of 200 mg doxycycline.

	MS	BM	IS	ML	Average
Microgram/ml	60	52	50	32	49
Excretion in mg	13.2	9.9	15.5	16.0	13.6
Excretion in per cent of the intake	6.6	5.0	7.8	8.0	6.8
Doxycycline clearance in per cent of creatinine clearance ...	13.9	11.7	11.2	15.5	13

amount of 1000 mg administered. It should be noted that the collection of urine was completed 24 hours after the last dose of doxycycline and hence only the excretions during the first 24 hours of the last dose, was collected.

The 8-hour doxycycline clearance determination in group I showed this to be 13 per cent of the creatinine clearance. The 96-hour clearance determination in group II showed an average doxycycline clearance of 28 ml/minute (table 5).

Determined within the first 24 hours after administration of the drug, an average half-life period of 11.6 hours was found in group I.

The results of our investigations of the antibacterial effect of doxycycline and oxytetracycline as well as of the comparison between the *in vitro* activity of the two drugs can be seen in table 6 and 7 and in fig. 4.

Table 6 represents the MIC-values of doxycycline and oxytetracycline.

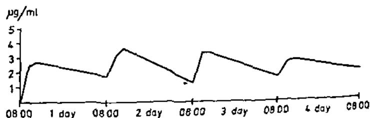


Fig. 2. Group II. Average concentration of doxycycline in the serum in 5 volunteers after the intake of 400 mg doxycycline at 8 a.m. on the first day and 200 mg at 8 a.m. on the 2nd, the 3rd and on the 4th day.

Table 3.

Group II. Serum concentrations in 5 volunteers after the intake of 400 mg doxycycline at 8 a.m. on the first day and 200 mg at 8 a.m. on the 2nd, the 3rd and the 4th day. μg doxycycline/ml serum.

Time	I. day			II. day			III. day			IV. day			V. day
	08.00	10.30	12.30	08.00	10.30	12.30	08.00	10.30	12.30	08.00	10.30	12.30	08 00
MSø (57 kg)	0	2 5	3 0	2.7	3.5	5.0	1.7	3.2	4.2	2.1	3.7	3.0	2.3
NH (52 kg)	0	2.7	3.2	2.7	3.8	4.2	1.8	3.5	3.2	1.6	2.8	3.7	2.1
MSk (59 kg)	0	2.7	3.0	1.3	3.0	3.0	1.0	3.5	3.2	1.1	2.5	2.5	2.1
ML (66 kg)	0	2.1	2.1	0.5	2.5	2.3	0.6	3 0	2.5	0.8	2.3	2.5	1.4
UP (56 kg)	0	2.7	2.5	1.9	3.0	3.9	1.6	3.9	3.9	1.8	2.3	3.0	2.1
Average	0	2.5	2.8	1.8	3.2	3.7	1.3	3.4	3.4	1.5	2.7	2.9	2.0
Standard deviation		0.23	0.44	0.94	0.50	1.10	0.91	0.11	0.66	0.52	0.58	0.49	0.34

Table 4.

Group II. Concentration and excretion of doxycycline in the urine in 4 volunteers after the intake of 400 mg doxycycline at 8 a. m. on the *first day and 200 mg at 8 a. m.* on the 2nd, the 3rd and the 4th day.

Time	I. day			II. day			III. day			IV. day			Total
	08–14	14–20	20–08	08–14	14–20	20–08	08–14	14–20	20–08	08–14	14–20	20–08	
MSø (57 kg) µg/ml	90.4	60.0	100.0	200.0	150.0	177.1	64.8	100 0	100.0	50.0	160.0	45.0	
excretion in mg	10.4	8.4	20.0	28.0	24.0	81.5	36.3	26.0	56.0	22.5	36.0	30.0	379.1
UP (56 kg) µg/ml	90.0	75.0	100 0	175.0	180.0	100.0	190.0	200.0	170.0	190.0	160.0	50.0	
excretion in mg	7.2	15.0	40.0	38 5	45.0	34 0	32.3	24.0	71.4	47.5	36.8	35.5	427.2
NH (52 kg) µg/ml	74.7	35 0	100.0	127.3	40.0	95.0	90.0	110.0	200.0	125.0	200.0	55.0	
excretion in mg	15.7	19.7	40.0	37.5	14.4	41.8	40.5	77.0	50.0	27.5	46.0	22.0	432.1
MSk (59 kg) µg/ml	116.1	74.6	92.1	150 0	80.0	62.2	120.0	100.0	94.8	90.0	75.0	59.2	
excretion in mg	15.1	20.6	50.4	45.0	33.6	43.2	28.8	24.0	68.4	11.7	61.5	27.0	429.3
Average excretion in mg	12.1	15.9	37.6	37.3	29.3	50.1	34.5	37.8	61.5	27.3	45.1	28.6	416.9
Standard deviation	4.3	5.5	12.7	6.9	13.1	21.1	5.0	26.1	10.1	15.3	11.8	5.6	10.9

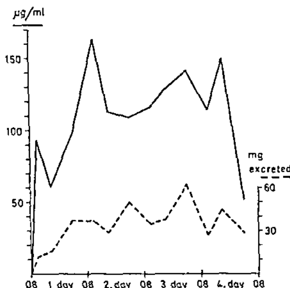


Fig. 3. Group II. Average excretion and concentration of doxycycline in the urine in 4 volunteers after the intake of 400 mg doxycycline at 8 a. m. on the first day and 200 mg at 8 a m. on the 2nd, the 3rd and the 4th day.

From the table it appears that on the whole doxycycline had a higher *in vitro* activity against the 211 strains of bacteria examined than had oxytetracycline. This activity is evident both against the relatively few, highly sensitive gram-negative rod-shaped bacteria, against the somewhat more frequently occurring sensitive gram-positive cocci, and against the more resistant strains: 51 per cent and 19 per cent of all the gram-negative strains were resistant to 100 µg/ml oxytetracycline and doxycycline, respectively. Among the gram-positive isolates 21 per cent were found to be resistant to 100 µg/ml oxytetracycline,

Table 5.

96-hours doxycycline clearance in 4 volunteers.

	ml/minute
MSø	22.1
NH	26.7
MSk	34.0
UP	28.6
Average	27.8
Standard deviation	3.3

Table 6.

MIC-values of doxycycline and oxytetracycline. *In vitro* activity against 211 frebly isolated strains. MIC µg/ml.

			0.78	1.56	3.12	6.25	12.5	25	50	100	>100	Total
E. coli		Dox.	1	10	19	14	5	6	10	15	7	87
		Ox.	1	5	12	19	8		4	3	35	
Klebsiella		Dox.		1	1	3	4	2	12	4	4	30
		Ox.			1	4	2	1	1	1	20	
Proteus		Dox.				1		1	1	6	16	25
		Ox.					1	1	1	3	19	
Other Enterobact.		Dox.			4	2	4	3	2	4	3	22
		Ox.			3	1	4	1	2	1	10	
Ps. aeruginosa		Dox.						1			1	2
		Ox.								1	1	
Total of gram neg. rods.	Number	Dox.	1	11	24	20	13	12	25	29	31	166
		Ox.	1	5	16	24	15	3	8	9	85	
	Per cent	Dox.	<1	7	14	12	8	7	15	17	19	
		Ox.	<1	3	10	14	9	2	5	5	51	
Str. faecalis		Dox.	5	2		4	4	6	9	2		32
		Ox.	2	2	2		2	2	3	6	13	
Staph. aureus		Dox.						2	3			5
		Ox.								1	4	
Other gram-pos. cocci		Dox.	1	1			2	1	3			8
		Ox.		1		1	1			1	4	
Total of gram-pos. cocci	Number	Dox.	6	3		4	6	9	15	2		45
		Ox.	2	3	2	1	3	2	3	8	21	
	Per cent	Dox.	13	7		9	13	20	33	4		
		Ox.	4	7	4	2	7	4	7	18	47	

Dox. = doxycycline.
Ox. = oxytetracycline.

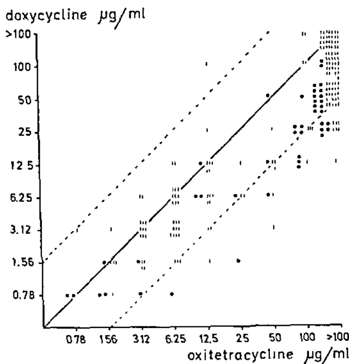


Fig. 4. *In vitro* activity against 211 freshly isolated bacterial strains. Comparison between the MIC-values of doxycycline and oxytetracycline.
I = gram-negative rods
• = gram-positive cocci
The two dotted lines indicate a difference in the concentration of 4 times.

whereas no strains were found to be resistant to doxycycline in this concentration.

A comparison between the bacteriostatic effects of the two tetracyclines on the strains, still assessed at the MIC-level, is shown in fig. 4. Doxycycline was more active than oxytetracycline in 38 per cent of the strains (80 out of the 211 strains tested). Only 2 isolates (1 *escherichia coli* and 1 *proteus mirabilis*) were more sensitive to oxytetracycline than to doxycycline (< 1 %). In 129 strains (61 %) there was no significant difference between the MIC of doxycycline and that of oxytetracycline.

In table 7 the distribution of the strains more sensitive to doxycycline than to oxytetracycline is shown. It appears that on the whole, gram-positive cocci as compared with gram-negative rods showed greater sensitivity to doxycycline than to oxytetracycline. Thus, out of a total of 45 gram-positive strains, 31 (67 %) were more sensitive to doxycycline, whereas in the gram-negative strains, only 48 out of 166 showed such sensitivity.

Table 7.

The distribution of the strains in which doxycycline, assessed at the MIC-level, shows a greater *in vitro* activity than oxytetracycline (a difference in the growth of at least two tubes of the dilutions).

	Doxycycline more active than oxitetracycline	Total number of strains	Per cent
E. coli	25	87	29
Klebsiella	15	30	50
Proteus	2	25	8
Other enterobact.	6	22	27
Ps. aeruginosa	1	2	50
Str. faecalis	21	32	66
Staph. aureus	4	5	80
Other gram-pos. cocci	*6*	*8*	75
Total number	80	211	38

Discussion

The single dose test (table 1) as well as the multiple dose test (table 3, fig. 2) show that with the low dosage (200 mg) given once every 24 hours, it is possible to keep an average minimum concentration in the serum above 1 µg/ml. Our investigation is thus in accordance with the findings obtained by others (DIMMLING 1967; FABRE *et al.* 1967). From the literature it appears that no previous determination of the serum and urine concentration has been performed with the dose used in the present study group II. Compared with DIMMLING'S investigation (1967), the high initial dose in the present investigation seems to be reflected in the minimum values, which in our material were higher than those found in DIMMLING'S (1967) multiple dose test with 200 mg every 24 hours.

In order to evaluate the sensitivity of the strains of bacteria to doxycycline and oxytetracycline, MIC-determinations were used. As appears from table 6, the material showed considerable variations. Nevertheless, the results clearly show that, taken as a whole, pathogenic gram-negative rodshaped bacteria are not very sensitive to the two tetracyclines used (87 and 105 out of 166 strains were resistant to doxycycline and oxytetracycline, respectively, in concentrations less than 25 µg/ml). As to the gram-positive cocci, only the group of *streptococcus faecalis* could be evaluated with any certainty. The other gram-positive strains occurred in such small numbers that it was impossible to assess the relative sensitivity for the two agents. The *streptococcus faecalis* strains were also often rather resistant to the antibiotics investigated: 17 and 24 of the 32 isolates were not sensitive to doxycycline and oxytetracycline, respectively, in concentrations less than 25 µg/ml.

Thus, with regard to the non-differentiated material the present results deviate from those of FABRE *et al.* (1966), of REBER (personal communication 1967), and from MONNIER & BOURSE (1967). The results of the two latter studies cannot, however, be compared directly with our own. REBER (personal communication 1967) used the disc method for the test, and found a considerable number of sensitive strains, but no definite difference between doxycycline and oxytetracycline with regard to their activity against most of the groups of bacteria investigated.

MONNIER & BOURSE (1967) used laboratory strains and found most of them sensitive (MIC 1.6 μg/ml or less against 17 out of 21 strains). The investigations of FABRE *et al.* (1966) can be related to ours, as these workers used newly isolated strains and MIC-determinations in about the same concentrations as our own. They found 114 and 109 out of 294 strains sensitive to doxycycline and oxytetracycline, respectively, in concentrations of 1 μg/ml or less. The high number of resistant strains of bacteria found in the present study is probably due to the fact that the strains originated from patients who had previously been treated with various antibiotics for periods ranging from 2 months to 2 years, due to recurrent urinary tract infections.

The comparison between doxycycline and oxytetracycline (table 6 and fig. 4) conclusively demonstrates that doxycycline on a weight basis has a greater antibacterial activity *in vitro* than oxytetracycline, particularly against gram-positive bacteria. Thus the present material confirms the previous reports on the subject (FABRE *et al.* 1966; MONNIER & BOURSE 1967).

Based on the present material it can be concluded that in the treatment of urinary tract infections of the chronic type, tetracyclines are seldom the drug of choice.

REFERENCES

Dimmling, T.: Bakteriologische Untersuchungen über Resorption und Exkretion sowie über das Wirkungsspektrum von Alpha-6-Desoxyoxytetracyclin (Doxycyclin). *Med. Klin.* 1967, 62, 1269–1276.

Fabre, J., I. S. Pitton & J. P. Kunz Distribution and excretion of doxycycline in man. *Chemotherapia* 1966, 11, 73–85

Fabre, J., J. S. Pitton, C. Virieux, F. L. Laurencet, J. P. Bernhardt & J. C. Codel: La doxycycline: absorption, distribution et excrétion d'un nouvel antibiotique a large spectre chez l'homme. *Schweiz. med. Wschr.* 1967, 97, 915–923.

Kirschbaum, A., J. Kramer & M. A. Garth· Uniform preparation of microbial suspensions for antibiotic assays. *Antibiot. and Chemother.* 1962, 12, 545–550.

Kunin, G. M., A. C. Dornbush & M. W. Finland· The distribution and excretion of four tetracycline analogues in normal males. *J. clin. Invest.* 1959, 38, 1950–1963.

Mighardi, J. R. & M. Schach von Wittenau: *Pharmacokinetic properties of doxycycline.* Medical Research Laboratories, Chas. Pfizer and Co., Connecticut, U. S. A. 1966.

Monnier, J. & B. Bourse: Activité antibacterienne *in vitro* et concentrations sanguines de la doxycycline. *5th International Congress of Chemotherapy.* Vienna, 1967.

Rosenblatt, J. E., J. E. Barrett, J. L. Brodie & W. M. M. Kirby: Comparison of *in vitro* activity and clinical pharmacology of doxycycline with other tetracyclines. *6th Interscience Conference on Antimicrobial Agents and Chemotherapy*. Philadelphia, 1966.
Vesterdahl, J.: *Måling og standardisering af penicillin*. Disputats, Copenhagen, 1947.
Williamson, G. M.: Laboratory studies on doxycycline. *International Symposium on Doxycycline*. Buenos Aaires, 1967.
Winkler, E. & H. Weih: Doxycyclin, Ergebnisse von Basisuntersuchungen. *Med. Klin* 1967, 62, 1257–1262.

Acta pharmacol. et toxicol. 1971, 29, 327–338.

From the Department of Pharmacology, University of Oslo,
Blindern, Oslo, Norway

The Adrenergic Receptors in Isolated Rabbit Stomach Muscle

By

J. F. W. Haffner

(Received September 15, 1970)

Abstract: The effects of stimulation of the adrenergic α- and β-receptors in muscle from the antrum and fundus of the rabbit stomach have been studied. The muscular activity was markedly different in preparations from the two parts. In the muscle from the antrum rhythmic contractions generally occurred, while alterations in the tone were small and infrequent. In the muscle from the fundus, rhythmic contractions were rarely seen, but considerable variations in tone occurred in response to drugs. β-receptor stimulation invariably gave inhibitory effects, but the effect of α-receptor stimulation in longitudinal and circular antrum strips, and in longitudinal fundus strips, depended on the existing muscular activity when the drug was added, high activity favouring inhibitory effects and low activity favouring excitatory effects. In a few antrum strips low concentrations of α-adrenergic agents increased the frequency of the rhythmic contractions while higher concentrations inhibited them. In circular strips from the fundus α-receptor stimulation gave only excitatory responses. The effect of the adrenergic agents were apparently due to a direct influence on the muscle, as the quality of the responses was unaffected by pretreatment with atropine, hexamethonium and tetrodotoxin, by the presence or absence of mucosa, or by cold storage for 24 hours.

Key-words: Adrenergic receptors – gastric motility – gastric smooth muscle.

Adrenergic agents generally reduce tension and inhibit rhythmic contractions in gastro-intestinal smooth muscle. According to Ahlquist (1948) and Levy & Ahlquist (1967) both α- and β-adrenergic receptors subserve inhibition in the intestines. But α-receptors which mediate excitation in gastro-intestinal muscle have also been reported (see Lee 1970). Excitatory responses to adrenergic stimulation are most easily elicited in the sphincter regions, but can also be obtained in other parts of the intestines.

It is known (Brown & McSwiney 1926; Furchgott 1967) that adrenaline may produce both excitatory and inhibitory effects in the rabbit stomach, but until recently excitatory effects had not been found in the stomach of any

other species. In 1968, however, BAILEY reported the existence of excitatory α-adrenergic receptors in the guinea-pig stomach, and this has later been confirmed by GUIMARÃES (1969). Stimulation of the α-receptors in isolated human stomach muscle generally produce inhibitory effects (BENNETT & WHITNEY 1966), but excitation can also be obtained (HAFFNER *et al.* 1969). In an attempt to gain more information about the factors which govern the adrenergic response in the stomach, we have studied the effects of adrenergic agents on isolated rabbit stomach muscle, which is known to give predominantly excitatory responses to adrenaline (BROWN & MCSWINEY 1926). The effects of α- and β-receptor stimulation in circular and longitudinal muscle strips from the antrum and fundus have been studied, and an attempt has also been made to determine the role of the nervous elements in the strips, by comparing the adrenergic responses before and after treatment with atropine, hexamethonium and tetrodotoxin. The effects of removal of the mucosa, and of cold storage for 24 hrs have also been studied.

Material and Methods

Rabbits of either sex weighing two to three kg were used. The animals were stunned by a blow on the occiput and bled to death by cutting the carotid artery. Immediately after death the stomach was removed and full thickness strips, 2–3 mm wide and 2–3 cm long, were cut circularly or longitudinally from the muscle coats of the antrum or fundus. Some strips were removed with the overlying mucosa attached, but from most of the preparations the mucosa and submucosa were removed. Four to eight fresh strips were taken from the stomach of each animal, the remaining part of the stomach being stored overnight in modified Krebs solution (see below) at 4°. Twenty-four hours old strips were prepared in the same way on the next day. Twenty-four rabbits were used for this investigation, and a total of 159 strips were investigated, 79 being taken from the antrum (42 longitudinal and 37 circular) and 80 from the fundus (47 longitudinal and 33 circular).

The strips were mounted in organ baths containing 50 ml modified Krebs solution at 37°, aerated with 95 % O_2 and 5 % CO_2. The Krebs solution contained (mM) Na^+ 136.9, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 133.6, and glucose 11.5. Muscle tension was recorded isometrically with Grass transducers (Model FT03) connected to a Grass Polygraph. The strips were allowed about one hour in the organ bath for equilibration before the experiments were started. The drugs tested were left in the bath for three to six minutes until maximal responses were obtained. The bath was then completely emptied and refilled with fresh solution. When α- or β-blocking agents were used, they were added to the bath at least 15 min. before the adrenergic agents. The interval between each new addition of drugs varied from 5 to 30 min. as a stable curve was required before each test.

The drugs tested were: adrenaline bitartrate, isoprenaline sulphate, phenylephrine (metaoxedrinum NFN) HCl, methoxamine HCl, phenoxybenzamine (benzylytum NFN) (dibenyline), phentolamine (regitin® Ciba), propranolol (proprasylytum NFN) (inderal® ICI), acetylcholine chloride, atropine sulphate, hexamethonium bromide and tetrodotoxin (Sankyo).

Results

The rabbit stomach consists of two macroscopically different parts; the fundus and the antrum, and an intermediate transitional zone – the corpus. Preparations from the fundus and antrum differ both in the type of muscular activity and in their responses to drugs, they are therefore treated as two separate groups. The characteristics of preparations from the corpus appeared to depend on the location from which they were taken – as would be expected in a transitional zone. Strips from the corpus are therefore excluded from this study.

Muscular activity.

As in other types of gastrointestinal muscle, the activity in rabbit stomach muscle can be described as phasic (= rhythmic contractions) and tonic (= relatively slow reversible changes in tension, = "state of contracture").

In the *antrum* preparations the phasic activity was most marked, and this activity occurred in all preparations which responded to acetylcholine or adrenergic agents. The effects of the various drugs were most easily seen as

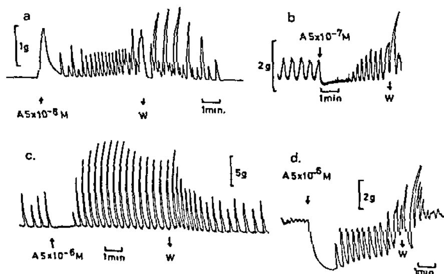


Fig 1. The effect of adrenaline (A) on antrum strips:
a: Inactive strip; a transient increase in tension is followed by the onset of rhythmic contractions.
b: Active strip; rhythmic contractions are inhibited, and there is a slight loss in tension.
c: A biphasic effect; after the initial inhibition, the amplitude of the contractions is increased.
d: A tetanically contracted strip relaxes and rhythmic contractions become apparent.

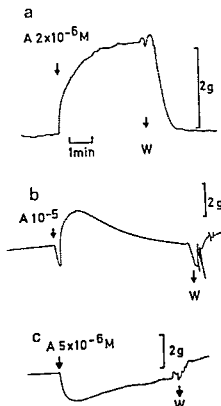


Fig. 2. Adrenaline (A) induces a sustained increase in tension in a relaxed longitudinal fundus strip (a), a biphasic change in tension in a moderately contracted (b), and a sustained loss of tension in a strongly contracted preparation (c).

changes in the rhythmic contractions. In strips from the fundus on the other hand, the activity was mainly tonic, contractions were seen in some preparations, but they were small and irregular. The effects of the drugs were seen as reversible changes in tension (= alterations in state of contracture).

The effect of adrenaline.

The effect of adrenaline (10^{-9}–10^{-5} M) varied according to the existing activity in the preparations.

Antrum. In inactive strips, adrenaline either had no effect or induced an excitatory response. In most preparations rhythmic contractions were initiated, often preceded by a transient tonic response (fig. 1a), and in a few instances only the tonic response occurred.

When adrenaline was added to strips which were contracting rhythmically, an inhibition occurred (fig. 1b). In a few preparations the effect appeared to

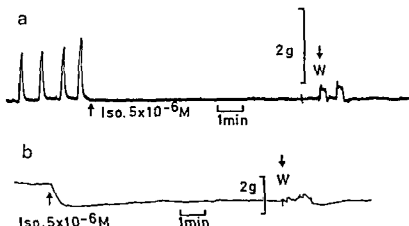


Fig 3. Beta-receptor stimulation with isoprenaline (Iso) leads to inhibition of rhythmic contractions in a strip from the antrum (a), and to a sustained loss of tension in a circular strip from the fundus (b) (the same response is obtained in longitudinal strips).

be biphasic (fig. 1c); after the initial inhibition the contractions were of a higher frequency and amplitude than before. When the preparations were in a state of tetanic contraction, adrenaline reduced the tension and the rhythmic activity again became apparent (fig. 1d).

No difference was found between longitudinal and circular preparations from the antrum.

Fundus. The effect of adrenergic stimulation differed in longitudinal and circular preparations. In *longitudinal* fundus strips which were completely relaxed adrenaline gave a sustained increase in tension (fig. 2a), in preparations which were moderately contracted the effect was biphasic (fig. 2b), and

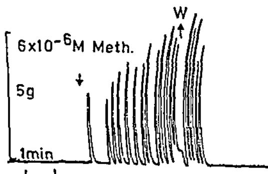


Fig. 4. Alpha-receptor stimulation with methoxamine (Meth) initiates rhythmic contractions in a strip from the antrum.



Fig. 5. In a moderately active antrum strip 5×10^{-7} M phenylephrine (P) increases the frequency of contractions, while higher concentrations inhibit the contractions.

in preparations which were already markedly contracted the effect was purely inhibitory (fig. 2c). The effect on *circular* strips was nearly always excitatory, but the magnitude of the response appeared to depend on the existing state of contracture.

Alpha- and beta-receptors.

The response to adrenaline is composite since this drug stimulates both the α- and β-receptors, hence it was necessary to study the effect of stimulation of each receptor separately. Phenylephrine (10^{-7}–10^{-5} M) and methoxamine (10^{-7}–10^{-5} M) were used to stimulate the α-receptors and isoprenaline (10^{-8}–10^{-5} M) to stimulate the β-receptors. In addition the effects of the α-receptor blocking agents, phenoxybenzamine (10^{-6}–10^{-5} M) and phentolamine (10^{-6}–10^{-5} M), and the β-receptor blocking agent propranolol (10^{-7}–10^{-5} M) were studied.

β-receptor stimulation, whether by isoprenaline or by adrenaline after α-receptor blockade, always inhibited the phasic and tonic activity where such activity was present (fig. 3), but had no effects on inactive preparations.

The effects of *α-receptor stimulation* were as variable as those of adrenaline, and also appeared to depend on the existing activity in the preparations. *In the inactive antrum strips α-receptor stimulation frequently initiated rhythmic contractions (fig. 4), whereas such contractions were inhibited in active preparations. The same dose of α-receptor stimulant was capable of producing these different responses in the same strips, the effect depending on the state of activity when the drug was added. In 6 strips in which the rhythmic activity could be described as moderate, low concentrations (10^{-7}–10^{-6} M) of phenylephrine or methoxamine increased the frequency of the contractions while higher concentrations (5×10^{-6}–5×10^{-5} M) inhibited the activity* (fig. 5). In the remainder of the preparations the quality of the response was independent of the concentration of α-receptor stimulant, but the magnitude of the responses was dose-dependent. The manner in which the antrum strips had been prepared seemed to be of no importance for the quality of the response.

The response to α-receptor stimulation in the *fundus strips* depended both on the existing state of contracture and on the direction in which the strips

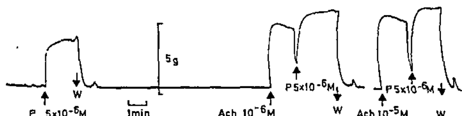


Fig. 6. Phenylephrine (P) gives a purely contractile effect in a longitudinal fundus strip which is completely relaxed, but when the strip is contracting in response to acetylcholine (Ach) a transient relaxation precedes the contraction. The magnitude of the two components depend on the existing tension at the time at which phenylephrine is added.

had been cut. In longitudinal strips a transient loss of tension was followed by a more sustained increase. The magnitude of the two components depended on the existing activity (fig. 6). In circular fundal strips on the other hand, α-receptor stimulation nearly always gave a sustained increase in tension. Even when the tension was very high the response was either excitatory or there was none.

Neither the excitatory nor the inhibitory effects of α-receptor stimulation were blocked by β-receptor blockade (28 preparations) while α-receptor blockade prevented both effects (19 preparations).

The effect of removal of the mucosa.

The mucosa was left attached to the muscle in 20 of the 79 antrum strips, and in 25 of the 80 fundus strips investigated. The effects of the adrenergic agents did not differ in preparations with and without mucosa; β-adrenergic stimulation with isoprenaline or with adrenaline in the presence of propranolol invariably gave inhibitory effects, while the effects of α-receptor stimula-

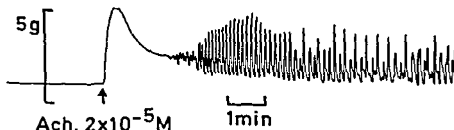


Fig. 7. A typical response to acetylcholine (Ach) in a previously inactive antrum strip; a transient increase in tension precedes the onset of rhythmic contractions. (Compare fig. 1a).

tion with phenylephrine or methoxamine, or with adrenaline in the presence of phenoxybenzamine or phentolamine, depended on the existing activity in all the strips other than the circular fundal, where the effect was always excitatory.

The effect of cold storage.

Thirty-five of the 79 antrum strips, and 40 of the 80 fundus strips were investigated after 24 hours cold storage. The effect of the adrenergic agents appeared to be the same in both the fresh preparations and in the preparations which had been stored.

The effects of acetylcholine.

The character of the response to acetylcholine in the various strips was determined in order to compare it with the response to the adrenergic agents. Acetylcholine (10^{-8}–10^{-4} M) always induced excitatory effects, but these varied in character. In all the *antrum* strips, a transient increase in tension occurred, lasting from ½–4 min. In addition rhythmic contractions were initiated or augmented in all the strips which had exhibited this type of activity earlier in the experiments, and also in a few preparations which had been totally unresponsive to adrenergic stimulation. A typical response is shown in fig. 7.

In the strips from the *fundus*, acetylcholine invariably induced a sustained increase in tension (see fig. 6), but no contractions.

In all the preparations acetylcholine was a far more potent and effective excitatory agent than either adrenaline, phenylephrine or methoxamine; the threshold for acetylcholine was lower than for the other drugs when related to molar concentrations, while the maximal contraction which could be produced by acetylcholine was greater than that produced by the other drugs.

The effects of atropine, hexamethonium and tetrodotoxin.

In order to determine whether the effects of adrenergic stimulation were due to a direct influence on the smooth muscle, or whether they were totally or partly mediated through nervous tissue, the effects of atropine, hexamethonium and tetrodotoxin on the adrenergic responses were studied, and compared with the effects on the response to acetylcholine.

Atropine (3×10^{-8}–3×10^{-7} M) had a slight inhibitory effect. A small depression of phasic and tonic activity occurred with the first addition, but this did not occur later. Atropine blocked the effect of acetylcholine completely (12 strips), but appeared to have no effect on the response to the adrenergic agents, whether excitatory or inhibitory.

Hexamethonium. It was thought that the dual effect (excitation/inhibition) of α-receptor stimulation might be due to stimulation of both the intramural

ganglia and smooth muscle. Fourteen preparations were therefore treated with hexamethonium (1.5×10^{-6} M) in order to block the ganglia. This did not alter the adrenergic responses. The effect of acetylcholine was also unaltered.

Tetrodotoxin. In an attempt to exclude all nervous influence, the effect of pretreatment with tetrodotoxin was studied. Twenty-five preparations were exposed to tetrodotoxin in concentrations from 10^{-7} to 10^{-6} g/ml for up to 20 minutes. The drug had no effect on the rhythmic activity or tension, nor did it in any way affect the adrenergic response, whether excitatory (20 preparations), biphasic (3), or inhibitory (2). The response to acetylcholine was also unaltered.

Discussion

In the present investigation the adrenergic β-receptors in the rabbit stomach muscle were found to mediate only inhibitory effects, whereas stimulation of the α-receptors gave both excitatory and inhibitory responses. This corresponds to the effects produced by stimulation of the adrenergic receptors in the guinea-pig (BAILEY 1968; GUIMARÃES 1969) and human stomach muscle (HAFFNER *et al.* 1969). The effect of stimulation of the α-receptors in guinea-pig stomach preparations depend on the existing activity at the time at which the drug is added, a high activity favouring inhibitory responses, while inactivity favours excitatory effects (GUIMARÃES 1969). In the present study this was also found to apply to rabbit stomach muscle preparations.

These results indicate that different degrees of activity may explain the qualitatively different results obtained in other investigations (e. g. BENNETT & WHITNEY 1966). Most investigators have used preparations in which the activity was high, i. e. either spontaneously or as a result of application of excitatory agents (e. g. high K^+, acetylcholine, carbachol, histamine) in the investigations on the effects of the catecholamines. This may be the reason why these investigators have obtained only inhibitory effects.

In our study of the effects of the adrenergic response in the human stomach muscle (HAFFNER *et al.* 1969), we found that in some preparations low concentrations of α-receptor stimulants induced excitatory effects, while higher concentrations of the same drug gave inhibitory responses. Similar effects were obtained in some of the antrum preparations used in the present investigation. This may indicate that the excitatory and the inhibitory α-adrenergic receptors have different thresholds, although no evidence for this was found in the remaining preparations. Since propranolol had no effect on the inhibition produced by the high concentrations of α-receptor stimulants, it is most unlikely that the inhibition would be due to β-receptor stimulation.

The muscular activity was different in the preparations from the different parts of the stomach. Rhythmic contractions were common in the smooth

muscle from the antrum, and the effect of the drugs were best seen as alterations in these contractions, while changes in basal tension were small or transient. In preparations from the fundus, on the other hand, the activity was mainly "tonic", the state of contracture of the strips being changed according to the drugs used. These differences reflect the functions of the two parts of the stomach; the antrum acts as a propulsive pump for the gastric contents, and helps in the mixing and mashing of the food, while the fundus mainly functions as a receptacle which maintains constant pressure in spite of varying volumes.

In the fundus there also seemed to be a difference between the longitudinal and circular strips; in the longitudinal strips α-receptor stimulation induced both excitatory and inhibitory effects, while the effect was almost exclusively excitatory in the circular strips. The functional significance of this is not known.

BAILEY (1968) found that removal of the mucosa from guinea pig stomach strips greatly enhanced the response to sympathomimetic amines. In the present study removal of the mucosa did not alter the adrenergic response qualitatively, which is in agreement with previous studies on intact and split strips from the fundus of the rat stomach (HORN & ZWEIFACH 1963). As the spontaneous activity varied in our preparations, it was difficult to study quantitative effects, and it cannot be excluded that removal of the mucosa gave minor quantitative alterations.

LUM *et al.* (1967) have reported that cold storage for 24 hours or more selectively reduces the responsiveness of the α-receptors in the rabbit intestine. In the present study the qualitative effects of adrenergic stimulation were the same in fresh preparations and preparations which had been stored for 24 hours, but again quantitative variations cannot be excluded.

Inactivation of the nervous tissue in intestinal preparations with tetrodotoxin has previously been shown to have no effect on the response to adrenergic agonists in isolated smooth muscle preparations (GERSHON 1967). In the present study the α-adrenergic responses were unaltered after the preparations had been treated with tetrodotoxin, hexamethonium or atropine. Both this and the lack of effect of removal of the mucosa indicated that the α-adrenergic agents acted directly on the adrenergic receptors in the smooth muscle cells.

The physiological implications of these findings are uncertain. The adrenergic nerves seem to terminate in the enteric plexi (NORBERG 1964), and it has been shown that there are α-receptors which regulate the liberation of acetylcholine from intramural cholinergic ganglia (PATON & VIZI 1969). As stimullation of the adrenergic nerves to the stomach has little or no effect when there is no cholinergic activity (JANSSON 1969), this would seem to indicate that the main function of the adrenergic nerves is to regulate cholinergic

activity. What then is the function of the adrenergic receptors in the smooth muscle cells? Two possibilities exist, they may either be activated by "overflow" of noradrenaline from adrenergic transmission sites in the submucous and intramuscular plexi, and thus act in conjunction with the adrenergic receptors in the cholinergic ganglia in the local response to sympathetic nervous stimulation, or they may be activated by circulating catecholamines and act as mediators of a more general systemic adrenergic response.

In the present investigation we did not succeed in quantitating the effects of the drugs. The threshold to the various drugs and the maximal responses varied in the individual strips, as did the relative potencies of the drugs. This may be due to the varying degrees of activity in the preparations, which was necessary for the determination of the effects of adrenergic stimulation at varying degrees of spontaneous muscular activity.

It would be interesting to study the effects of adrenergic stimulation, especially the effects of the α-adrenergic agents, with controlled variations of muscular activity, and such studies are now in progress in this laboratory.

Acknowledgements

The research reported in this communication has been sponsored partly by the Norwegian Research Council for Science and Humanities and partly by Norsk Medisinaldepots Fond.

REFERENCES

Ahlquist, R. P: A study of the adrenotropic receptors. *Amer. J Physiol.* 1948, **153**, 586–600.

Bailey, D. M.: Some effects of sympathomimetic amines on isolated smooth muscle preparations from the stomach of the guinea-pig. *Brit. J. Pharmacol* 1968, **34**, 204 P.

Bennett, A. & B. Whitney: A pharmacological investigation of human isolated stomach. *Brit. J. Pharmacol.* 1966, **27**, 286–298.

Brown, G. L. & B. A. McSwiney: Reaction to drugs of strips of the rabbit gastric musculature *J Physiol.* (Lond.) 1926, **61**, 261–267

Furchgott, R. F.: The pharmacological differentiation of adrenergic receptors. *Ann. N. Y. Acad. Sci.* 1967, **139**, 553–570.

Gershon, M D.: Effects of tetrodotoxin on innervated smooth muscle preparations. *Brit. J. Pharmacol.* 1967, **29**, 259–279.

Guimarães, S.: Alpha excitatory, alpha inhibitory and beta inhibitory adrenergic receptors in the guinea-pig stomach. *Arch. int Pharmacodyn.* 1969, **179**, 188–201.

Haffner, J. F. W., I. Liavåg & J. Setekleiv: Excitatory adrenergic receptors in the human stomach and pylorus. *Scand. J. Gastroent* 1969, **4**, 145–150.

Horn, L. & B. W. Zweifach. Some factors affecting the response of smooth muscle to chemical mediators *Angiology* 1963, **14**, 139–148.

Jansson, G.: Extrinsic nervous control of gastric motility. *Acta physiol. scand.* 1969, Suppl. 326.

Lee, C. Y.: Adrenergic receptors in the intestine. In: *Smooth muscle*. Eds.: Edith Bülbring *et al.* Edw. Arnold Ltd., London, 1970, pp. 549-557.

Levy, B. & R. P. Ahlquist: Adrenergic receptors in intestinal smooth muscle. *Ann. N. Y. Acad. Sci.* 1967, 139, 781-787.

Lum, B. K. B., R. D. Heilman & M. A. Gaunt: Selective loss of responsiveness to alpha adrenergic receptor stimulation after cold storage of the rabbit intestine. *Europ. J. Pharmacol.* 1967, 1, 109-113.

Norberg, K.-A.: Adrenergic innervation of the intestinal wall studied by fluorescence microscopy. *Int. J. Neuropharmac.* 1964, 3, 379-382.

Paton, W. D. M. & E. S. Vizi: The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea pig ileum longitudinal muscle strip. *Brit. J. Pharmacol.* 1969, 35, 10-28.

Acta pharmacol. et toxicol. 1971, 29, 339–358.

From the Research Laboratories of H. Lundbeck & Co. A/S,
Copenhagen, Denmark

Metabolism, Distribution and Excretion of Flupenthixol Decanoate in Dogs and Rats

By

A. Jørgensen, K. Fredricson Overø and V. Hansen

(Received October 13, 1970)

Abstract: The metabolism of flupenthixol decanoate administered in viscoleo® by intramuscular or subcutaneous injection has been studied by thin-layer chromatographic techniques after extraction of urine, faeces and organs from dogs and rats. Since only minute amounts of the compound itself were detected in the organs and a high hydrolytic activity of blood and organs was demonstrated by *in vitro* experiments, it was concluded that the compound is hydrolyzed in the organism. The flupenthixol thus formed was found to be metabolized by sulphoxidation and by dealkylation in the side chain following the same pattern as that previously shown for flupenthixol. The neuroleptic effect seen in the pharmacological studies is presumably due to flupenthixol, since this is quantitatively by far the most important substance found in brain extracts. In order to study the depot effect of the preparation, blood levels and excretion were followed after administration of the ^{3}H-labelled compound. These studies indicated, that a sustained release of drug from a depot was obtained. The presence of a relatively limited depot was demonstrated.

Key-words: Neuroleptic – sustained release preparation – kinetics – chromatography, thin-layer – radioisotopes.

The thioxanthene flupenthixol (fluanxol®) is well established in psychiatry as a neuroleptic drug without any sedative properties. A depot preparation (fluanxol® depot) suitable for intramuscular injection in oil has been obtained by the esterification of flupenthixol with decanoic acid. A prolonged neuroleptic effect of this preparation as compared to orally administered flupenthixol has been shown in pharmacological studies by MØLLER NIELSEN *et al.* (unpublished results) and FRANCK (1970) and in clinical trials by REMVIG *et al.* (1968), GJESTLAND (1970), ENERHEIM *et al.* (1970) and BILDE & MADSEN (1970).

The present publication describes studies performed in order to compare the pharmacological studies with the biochemical data and to determine the distribution, metabolism and excretion pattern of the depot preparation.

Materials and methods

A. Studies with unlabelled compound

1. Substance.

In flupenthixol (for formula see fig. 1) the side chain is attached to the ring structure with a double bond. Since the ring structure is asymmetrical the compound exists in two isomeric forms named α and β. In the depot preparation the pure α-isomer has been used, since this form has been shown to possess the highest biological activity.

2. Animals and dosage.

Adult pure bred Beagle dogs of either sex from our own stock were given 6 mg/kg of α-flupenthixol decanoate in viscoleo® (0.3 ml/kg) by injection in the posterior musculature of the thigh once a week for 6 months.

Rats, SPF, [Wistar/Af Han/Mö (Hann 67)] were given a subcutaneous injection of 10-14 mg/kg α-flupenthixol decanoate in viscoleo® (0.1 ml/rat) in the nape of the neck twice a week for 7 weeks.

3. Samples.

Twenty four hour samples of urine and of faeces were collected separately from the dogs after 10 days and after administration for 3 months. Samples of the liver, lungs, kidneys, brain, muscle (quadriceps femoris), fat and bile were obtained 9 days after the administration of the last dose. Urine and faeces were collected from the rats during the first 24 hours, after 4 weeks and also at the end of the study. Samples of organs were obtained 2 days after the last dose

4. Preparation of extracts

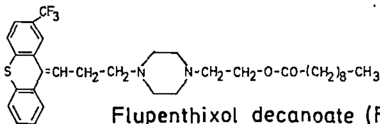
Samples of faeces and organs were homogenized 1 + 4 (weight/weight) in water before extraction.

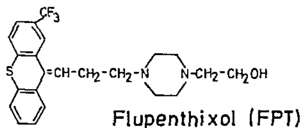
Simple extracts were prepared by extracting three times with the double volume of dichloroethane (DCE) at pH 10. Separation was achieved by centrifugation. The extracts were filtered through anhydrous sodium sulphate and evaporated to dryness under vacuum at 40°. The residue was dissolved in 1.0 ml of chloroform and used for thin-layer chromatography (TLC). After exhaustive extraction, hydrolysis was undertaken with β-glucuronidase, bacterial (Sigma), after which the samples were extracted as before

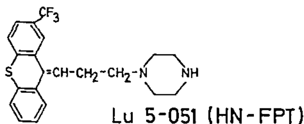
More purified extracts were obtained by introducing rinsing steps as illustrated in fig. 2. Following basic extraction with DCE, the organic extract was evaporated and the residue dissolved in 4 ml of 0.05 M acetic acid. The acid phase was extracted with petroleum benzene, which was subsequently divided into two portions one was prepared for TLC directly (c) – the other was evaporated and the residue redissolved in 5 ml of 5 M sodium hydroxide and placed in a boiling waterbath for 30 minutes for hydrolysis. The hydrolyzed sample was then extracted with DCE and prepared for TLC (b). The remaining acetic acid phase was adjusted to pH 10 and extracted with DCE. The extracts were prepared for TLC as above (a). All the extractions were made by shaking three times with a double volume of organic solvent. 50 ml of urine and 5–20 grams of organ or faeces were used for extraction. The residues were dissolved in 500 μl of chloroform of which 50–250 μl were spotted for TLC.

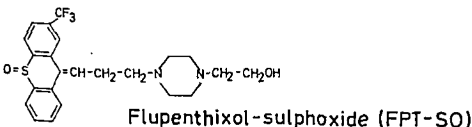
5. Thin-layer chromatography (TLC)

TLC was carried out on glass plates (20 × 20 cm) coated with a 250 μ thick layer of Silica Gel G. according to Stahl (Merck) After coating, the plates were activated at 110° for 30 min. and stored.









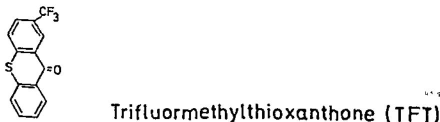


Fig. 1. Reference substances.

The following solvent systems were used

I	Isopentyl alcohol	10 ml
	Acetic acid	20 ml
	Water	20 ml
II	Propyl alcohol (n)	85 ml
	Water	15 ml
III	Propyl alcohol (n)	95 ml
	Water	5 ml
IV	Acetone	60 ml
	Heptane	40 ml
	Diethylamine	10 ml
V	Methanol	39.1 g
	Benzene	60.9 g

In some instances two-dimensional chromatography was carried out using rinsing systems such as chloroform : ether (85 : 15) or benzene in the first direction and one of the systems mentioned above in the second. The plate was thoroughly dried between the runs.

Concentrated sulphuric acid was used as the spray reagent. The plates were observed in UV-light.

The following substances were used as references (for formulas see fig. 1): α-flupenthixol decanoate (α-FPT-dec.), flupenthixol (FPT) (pure α and mixture of α and β), flupenthixol sulphoxide (FPT-SO), Lu 5-051 (HN-FPT) and trifluormethylthioxanthone (TFT). Rf-values for the reference substances are shown in table 1.

The sulphoxides of FPT-dec. and HN-FPT, which were not available as reference substances, were formed directly on the TLC-plate by applying 2 drops of 10 % H_2O_2 on top of a sample of FPT-dec. and HN-FPT, respectively.

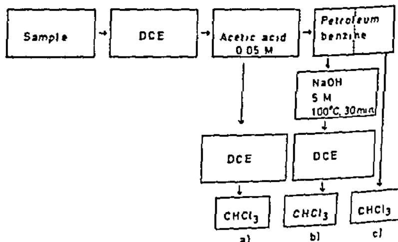


Fig. 2. Extraction procedure.

B. *Studies on labelled compounds.*

1. *Labelled compounds.*

2-trifluormethylthioxanthone (TFT) was tritium (T) labelled by catalytic exchange by New England Nuclear.

The specific activity of the raw product was 93.6 mci/mg. Thin-layer chromatography (TLC) revealed that not more than 62.5 % was TFT. At least 7 labelled impurities were found to be present.

TFT was purified by several recrystallizations from acetic acid and diluted 5 times with unlabelled substance. TLC showed that 91.5 % of the radioactivity followed TFT.

From TFT flupenthixol was synthesized in 3 steps and flupenthixol decanoate in a further step as shown in fig. 3. The first step consisted of a reaction with dimethylaminopropylmagnesiumchloride (DMAPMgCl) to give N 7057, which was purified to 98 % (radiochemical purity) by recrystallization from petroleum ether.

N 7057 was dehydrated in a mixture of acetic acid and hydrochloric acid to give N 796, which in turn was converted to FPT by amine exchange with hydroxyethylpiperazine (HEPPZ). *FPT was purified by recrystallization as base from petrol ether.* By this procedure pure α-isomer was obtained. The radiochemical purity of this compound was > 90 %.

α-FPT-dec. was synthesized by reaction of α-FPT with decanoylchloride (DECl). α-FPT-dec. was purified by recrystallization as an oxalate and controlled by TLC in several systems.

Two lots were made.

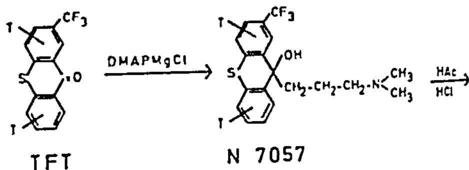
Lot no. 1: ^{3}H-α-Flupenthixol decanoate:

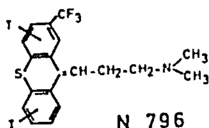
Specific activity: 578 μci/mg
Radiochemical purity: > 85 %
(About 8.5 % of TFT present).

Table 1.

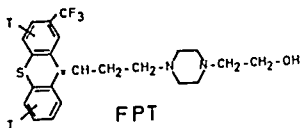
Rf-values in TLC.

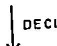
Solvent system	Reference substances				
	FPT-dec.	FPT	FPT-SO	HN-FPT	TFT
I	0.67	0.31 0.27	0.19	0.36 0.33	0.93
II	0.86	0.50 0.41	0.27 0.21	0.11 0.05	0.83
III	0.74	0.20	0.10	0.04	0.87
IV	0.95	0.60	0.50	0.35 0.26	0.87
V	0.94	0.76	0.66 0.61	0.27	0.94











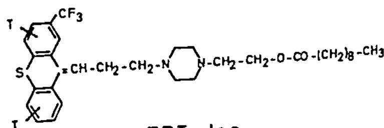


Fig. 3. Synthesis of tritium labelled FPT and FPT-dec.

Lot no. 2: ^{3}H-α-Flupenthixol:
Specific activity: 560 μci/mg
Radiochemical purity: > 90%

^{3}H-α-Flupenthixol decanoate:
Specific activity: 378 μci/mg
Radiochemical purity: > 95 %

^{3}H-α-FPT-dec. was prepared for intramuscular injection by dissolution in viscoleo® (20 mg/ml).

2. *Animal studies.*

a. *Rat study on blood levels and excretion.* Male Wistar/Af/Han/Mö (Han 67) rats, SPF, (115–130 g) were given an intramuscular injection of 5 mg ^{3}H-α-FPT-dec. (lot no. 1)/kg body weight into the left musculus extensor quadriceps femoris. At ½, 2 and 8 hours, and 1, 3, 6, 10, 16, 21 and 28 days after injection, two rats from each of the groups were killed under ether anaesthesia and blood samples were collected in tubes containing EDTA to prevent clotting. From the four rats killed 21 and 28 days after injection, 24 hour samples of urine and faeces were collected separately during the entire experimental period.

b. *Rat study on distribution.* Male Wistar/Af/Han/Mö (Han 67) rats, SPF, (205–250 g) were given an intramuscular injection of 4 mg ^{3}H-α-FPT-dec. (lot no. 2)/kg body weight in the left musculus extensor quadriceps femoris. At 6 hours, and 1, 4, 7 and 14 days after the injection, 2 animals were killed by exsanguination under ether anaesthesia. Blood samples were collected in tubes containing EDTA to prevent clotting and the brain, lungs, liver, heart, kidneys, spleen, epididymal fat and the left leg were removed. From the two rats killed on the 14th day after the injection blood samples were drawn from the orbital vein plexus at ½, 2, 6 hours, and 1, 2, 4, 7, 10 and 14 days after administration of the drug.

c. *Dog study on blood levels and excretion.* Six adult pure bred Beagle dogs of either sex (b.w. 9.4–11.5 kg) from our own stock, divided into three groups of one male and one female each, were used in the study. Two groups were given 2 and 6 mg ^{3}H-α-FPT-dec. (lot no. 2)/kg respectively by intramuscular injection in the posterior musculature of the thigh, while the third group, for comparison, was given an oral dose of 1 mg ^{3}H-α-FPT/kg as pure substance in a capsule. Blood samples were drawn by vein puncture at different times after administration of the drug. Urine and faeces samples were collected in the periods 0–1, 1–2, 3–4, 6–7, 10–11, 15–16 and 24–25 days respectively after administration of the drug.

3. *In vitro study on hydrolysis*

Two male Wistar/Af/Han/Mö (Han 67) rats, SPF, were bled under ether anaesthesia. The blood was collected in heparinized tubes and diluted with 4 volumes of 0.1 M phosphate buffer, pH 7.4. Brain, liver, lungs and kidneys were removed and homogenized in 4 volumes of the same buffer. To 12.5 ml of buffer, diluted blood and organ homogenates were added 26.5 μg of ^{3}H-α-FPT-dec. and the samples then incubated with shaking at 37°. Five ml samples were removed for analysis after 1 and 2 hours respectively.

4. *Preparation of samples for liquid scintillation counting.*

The radioactive samples were measured in a Beckman Liquid Scintillation Counter with automatic external standardization. The scintillators used were toluene: triton X-100 (2 : 1) containing 6 g/l PPO and 0.3 g/l dimethyl-POPOP for who

and for all other samples the dioxane-methanol-toluene-naphthalene mixture (dioxol) described by HERBERG (1960) and modified by replacing POPOP with dimethyl-POPOP, was used. 10 ml portions of the scintillators were used.

a. *Blood.* To 0.1 ml of whole blood were added 1 ml of concentrated NH_3-solution and 0.1 ml of 30 % H_2O_2-solution. After gentle shaking the sample was kept at room temperature for one hour and heated for another hour in an oven at 60°. After cooling the scintillator was added. 0.5 ml of serum was counted directly in dioxol scintillator.

b. *Tissues.* Homogenates containing 1 part by weight in 4 volumes of water were made of the brain, lungs, liver and kidneys. A 0.5 ml aliquot was added to the scintillator. The heart, epididymal fat, left leg and spleen were heated in 4 volumes of 0.5 N NaOH solution in sealed tubes on a water bath at 90–95° for 30 min. with occasional shaking. Aliquots of 0.5 ml were taken for counting. The remainder of the animal body, named carcass, was refluxed in 2 volumes of ethanolic 0.5 M-KOH solution for one hour. After cooling and centrifugation 0.2 ml of the supernatant was counted.

c. *Urine and faeces.* 0.2 or 0.5 ml of urine was counted directly. The samples of rat faeces were ground in a mortar to give a homogenous mixture. Faeces from the dog were homogenized in 2 or 3 volumes of water. 0.5 g of rat faeces and 2 g of homogenate of dog faeces were heated for an hour in sealed tubes in an oven at 70–80° with 1 ml of 60 % $HClO_4$-solution and 2 ml of 30 % H_2O_2-solution. After cooling the solution was neutralized with solid NaOH and mixed with 40 ml of cellosolve. 1 ml of this mixture was then counted.

5. Extraction and chromatography

Dog urine (2–4 ml), homogenates of dog faeces (corresponding to 1–2 g of faeces) and homogenates of rat organs (3–5 ml) were adjusted to pH 10 and extracted three times with 20–30 ml of DCE. The pooled extracts were concentrated to 3–5 ml at low pressure. One aliquot of the concentrate was counted and another subjected to TLC. The samples from the *in vitro* study were shaken with 20 ml of DCE following addition of 0.5 ml 2 N-NaOH solution. Aliquots of the DCE phases were chromatographed. The TLC was run on 5 × 20 cm glass plates prepared as described above. Solvent systems I, III and IV were used. After development of the plates the silica gel was scraped off automatically for each half centimeter into separate counting vials and the radioactivity determined.

Results

A. Studies with unlabelled compound.

Comments on the procedures.

When studying blind samples to which reference substances had been added, it was observed that partial decomposition of the substances occurs during the extraction and TLC-procedures. Thus minute amounts of FPT are formed from FPT-dec. The formation of negligible amounts of sulphoxides, TFT and some unidentified products was also observed. In addition some isomerisation of α-FPT occurs relatively easily during extraction and also during TLC, as demonstrated by two-dimensional TLC, using the same

solvent system in both directions. These facts have been kept in mind when assessing the dosed samples used.

By the simple extraction technique, nearly complete extraction of the added standards was achieved as judged by TLC. By the more complicated technique small amounts of the substances were lost. However, considerable amounts of impurities were eliminated, thus providing an extract much more suitable for TLC. In addition partial separation of FPT-dec. from the other compounds was accomplished by extracting the acetic acid phase with petroleum benzene (see fig. 2). In this step FPT-dec. is completely extracted together with minute amounts of FPT. The main part of FPT and all of the other metabolites, however, remain in the acid phase and appear in extract a, while FPT-dec. appears in extracts b (hydrolyzed to FPT) and c. The use of other methods for obtaining more pure extracts, as e. g. gel filtration was complicated by absorption of the compounds to the gel and also by the instability of the compounds.

Dog studies.

TLC of simple extracts of urine revealed the presence of yellow fluorescent spots corresponding to the reference substance FPT, and orange fluorescent spots corresponding to FPT-SO. The two metabolites were present in approximately equal amounts (about 40 m μg/ml). No other substances could be detected. Neither was there any further release of substances observed after hydrolysis. The same results were obtained at TLC of purified extracts.

In extracts of faeces, distinct yellow spots corresponding to FPT and HN-FPT were detected in approximately equal concentrations (about 0.5 μg/g). No other substances were observed.

Useful results with TLC on organs were obtained only by using purified extracts and two-dimensional TLC. In the a-extract of liver (see fig. 2) small amounts of FPT and very small amounts of HN-FPT and FPT-SO were observed. The c-extracts contained small amounts of FPT-dec. and extremely small amounts of FPT. When the contents of the latter extract had been submitted to hydrolysis only FPT was detected in the extract (b-extract). Thus the presence in the liver of FPT-dec., FPT, NH-FPT and possibly FPT-SO was demonstrated. The main compounds, FPT-dec. and FPT, were judged to be of the same order of concentration (approximately 25–100 m μg/g).

The same pattern of drug and metabolites was detected in the lungs, the amounts being of the same order as in the liver. In the kidneys, however, the amounts were very low. In the brain and muscle neither drug nor metabolites could be detected.

The bile was found to contain some drug material, the identification of which was impossible because of interference by the impurities.

The FPT detected by TLC in the samples mentioned above appeared as

two spots corresponding to the two isomeric forms of FPT, α and β, in solvent systems I and II. The two spots were of approximately equal size, thus indicating a larger degree of isomerisation than that normally occurring during the procedures. This seems to indicate that a certain degree of isomerisation takes place in the animal body.

Rat studies.

In extracts from rat urine FPT-SO was found to be the main compound. The concentration was estimated to be about 0.5 μg/ml. In addition FPT (approximately 0.3 μg/ml) and HN-FPT-SO were detected. By hydrolysis with β-glucuronidase further amounts especially of FPT, but also of FPT-SO, were released. Extracts of faeces were found to contain FPT and HN-FPT as the main metabolites. In addition the presence of very small amounts of FPT-SO was indicated.

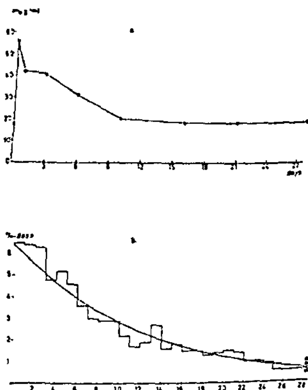


Fig. 4. a. Blood level of radioactivity in rats killed at different times after administration of 5 mg FPT-dec./kg intramuscularly. n = 2. *Ordinate:* Radioactivity expressed as mμg FPT-dec./ml.

b. Total excretion of radioactivity in 24 hour samples of urine and faeces from rats given 5 mg FPT-dec./kg intramuscularly.

Studies on extracts from organs revealed only the presence of very small amounts of FPT in the liver.

The contents of FPT detected by TLC in these samples were divided into two spots of about the same size, as was also the case in the dog material.

B. *Studies on labelled compounds.*

Rat study on blood levels and excretion. The radioactivity in rat blood expressed as mμg FPT-dec./ml blood following intramuscular injection of FPT-dec. is shown in fig. 4a. The curve shows a peak value 8 hours after injection and a rather slow decrease after this time. In fig. 4b the total excretion of radioactivity in the urine and faeces is shown. The exponential excretion curve estimated by a regression analysis using the method of least squares on the excretion data is also shown. The regression analysis gave an elimination half-life of 8 days for the total excretion of radioactivity. The total excretion within 28 days was 71.4 %, indicating that part of the radioactive substance was still in the animal body at the end of the study. The faecal excretion was highest being about seven times greater than the urinary excretion.

Rat study on distribution. The concentration of radioactivity in rat blood drawn from the orbital vein plexus is shown in fig. 5. The curve is very similar to that obtained from the above mentioned study in the rat (fig. 4a), with a peak value at 6 hours after injection. The tissue concentrations of radioactivity expressed as μg FPT-dec./g tissue are given in table 2. Apart

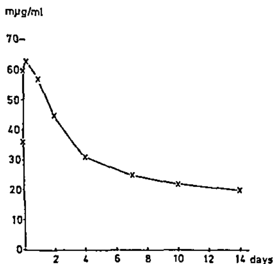


Fig. 5. Concentration of radioactivity in rat blood drawn from the orbital vein plexus (n = 2). Dose: 4 mg FPT-dec./kg intramuscularly. Ordinate: Radioactivity expressed as mμg FPT-dec./ml.

Table 2.

Concentration of radioactivity in rat tissues after 4 mg FPT-dec./kg intramuscularly (Expressed as μg FPT-dec./g and % of dose) (n = 2).

	6 hours		1 day		4 days		7 days		14 days	
	μg/g	%-dose	μg/g	%-dose	μg/g	%-dose	μg/g	%-dose	μg/g	%-dose
Blood	–	–	0.10	–	0.02	–	0.03	–	–	–
Brain	0.11	0.0	0.09	0.0	0.04	0.0	0.04	0.0	0.03	0.0
Heart	0.15	0.0	0.12	0.0	0.04	0.0	0.04	0.0	0.03	0.0
Lungs	1.3	0.2	1.5	0.3	0.2	0.0	0.2	0.0	0.1	0.0
Liver	1.6	1.7	1.5	1.6	0.3	0.3	0.3	0.4	0.2	0.3
Kidneys	0.6	0.1	0.6	0.1	0.1	0.0	0.2	0.0	0.1	0.0
Spleen	0.5	0.0	0.4	0.0	0.1	0.0	0.1	0.0	0.1	0.0
Epid. fat	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.0	0.0
Left leg	37.8	58.9	10.9	19.2	17.7	34.0	8.3	16.6	7.0	12.7
"Carcass"		20.9		14.2		1.8		2.3		1.4
Total		81.8		35.5		36.1		19.3		14.4

from the left leg in which the injection was performed, the highest concentrations were seen in the liver, the lungs, the kidneys and the spleen, while the concentrations in the brain and the blood were considerably lower. A maximum concentration was obtained 6 hours after injection in all the tissues except the lungs, in which a maximum concentration was found 24 hours after injection. As the decrease of radioactivity in the tissues was not exponential no half-life for the radioactivity in the tissues could be estimated. However, it should be stressed, that the concentration of radioactivity in the brain was still measurable 14 days after injection, when it was one third of the concentration found at 24 hours.

Relatively large variations were seen in the extractability of the radioactivity from the organs. This ranged from 11–59 % for the brain, 24–69 % for the liver, 67–92 % for the kidneys and 38–77 % for the lungs. The per-

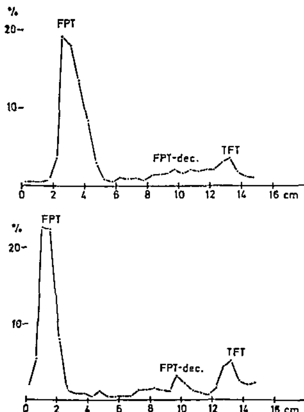


Fig. 6. Thin-layer chromatograms of brain extract from a rat killed 7 days after administration of 4 mg FPT-dec./kg intramuscularly. Abscissa: Distance from the starting point. Ordinate: Amounts of radioactivity in per cent of total radioactivity on the plate. Upper: Solvent system I. Lower: Solvent system III.

centage of extractable radioactivity for all organs decreased with time after administration.

In the chromatograms of the extracts of the brain (see fig. 6) a large peak corresponding to FPT was seen. This peak amounted to 52–83 % of the extractable radioactivity in the first week, decreasing to 32–63 % at 2 weeks after administration. The presence of small amounts of FPT-dec. and some metabolites was also indicated. In addition the presence of TFT was indicated. This substance is probably not a metabolite, as it is formed spontaneously during the handling of samples.

The chromatograms of extracts of the liver, lungs and kidneys had the same general appearance. The peak corresponding to FPT was the largest, but it was less marked than in the brain. In addition to FPT the presence of FPT-dec., FPT-dec.-SO and FPT-SO together with very small amounts of other metabolites was indicated. A peak corresponding to TFT was always seen.

Dog study on blood levels and excretion. The concentration of radioactivity expressed as mμg parent substance/ml serum in the serum from dogs given FPT-dec. or FPT is shown in fig. 7. Following FPT-dec. peak concentrations are seen at 7 days after administration, while in the two dogs given FPT the peak concentrations are seen at 2 and 24 hours respectively.

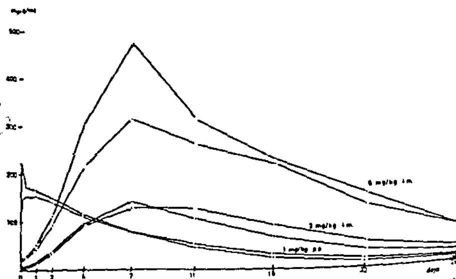


Fig. 7. Concentration of radioactivity in serum from dogs given *FPT orally* (1 mg/kg) or FPT-dec. by *intramuscular injection* (2 or 6 *mg/kg*) expressed as mμg of parent substance per ml serum.

Table 3.

Excretion of radioactivity in urine and faeces from dogs.

	1 mg FPT/kg p. o.				2 mg FPT-dec./kg i. m.				6 mg FPT-dec./kg i. m.			
Urine	%-dose/ ml × 10^3	%-dose	%-dose/ ml × 10^3	%-dose	%-dose/ ml × 10^3	%-dose	%-dose/ ml × 10^3	%-dose	%-dose/ ml × 10^3	%-dose	%-dose/ ml × 10^3	%-dose
0– 1 day	28.5	5.0	79.4	11.9	1.4	0.1	1.4	0.1	1.4	0.2	2.6	0.2
1– 2 days	13.0	2.0	8.1	2.0	1.6	0.2	2.0	0.2	0.9	0.3	1.9	0.6
3– 4 days	2.0	0.3	2.8	0.8	2.8	0.5	4.7	1.0	2.2	0.6	4.1	1.0
6– 7 days	1.2	0.2	1.2	0.3	3.7	0.4	5.4	0.9	4.8	0.6	8.1	2.2
10–11 days	0.6	0.1	0.7	0.2	1.7	0.4	1.6	0.3	1.5	0.5	2.2	0.5
15–16 days	0.4	0.1	0.3	0.1	1.1	0.2	1.1	0.1	1.2	0.2	1.4	0.2
24–25 days	0.2	0.01	1.1	0.2	0.7	0.1	0 6	0.04	0.9	0.1	0 8	0.1
Faeces	%-dose/ g × 10^3	%-dose	%-dose/ g × 10^3	%-dose	%-dose/ g × 10^3	%-dose	%-dose/ g × 10^3	%-dose	%-dose/ g × 10^3	%-dose	%-dose/ g × 10^3	%-dose
0– 1 day	360.3	8.9	625.6	53.4	12.9	0.5	3.5	0.3	–	–	20.8	1.6
1– 2 days	154 6	14.2	–	–	11.0	0.5	–	–	6.1	0.6	–	–
3– 4 days	11.1	1.6	11.4	0.8	34.7	1.9	57.0	3.6	41.0	4.6	56.8	5.7
6– 7 days	1.7	0.2	2.2	0.1	25.1	1.1	36.5	1.2	34.7	1.0	120.6	2.5
10–11 days	1.5	0.1	0.9	0.03	9.3	0.1	7.9	0.3	16.9	1.2	7.6	0.3
15–16 days	0.7	0.02	0.6	0.04	8.4	0.2	28.9	0.4	7.2	0.3	1.7	0.1
24–25 days	0.3	0.02	0.4	0.03	1.8	0.03	1.0	0.03	5.8	0.2	2.0	0.1

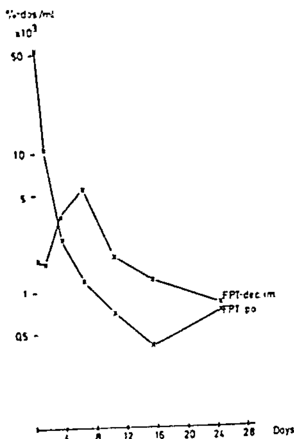


Fig. 8 Excretion of radioactivity in urine from dogs given FPT orally (2 dogs) or FPT-dec. intramuscularly (4 dogs)

The excretion of radioactivity in the urine and faeces is shown in table 3. Since large variations were seen in the amount of urine and faeces obtained from different dogs as well as in the same dog at different periods, the samples can not be considered as 24 hours collections. Therefore the percentage dose/ml urine and percentage dose/g faeces respectively have been considered instead of the percentage dose/time interval. The mean values for excretion in the urine (expressed as %-dose/ml) after administration of FPT-dec. (4 dogs) and FPT (2 dogs) are shown in a semilogarithmic plot in fig. 8. The curves show the same picture for excretion in the urine as that obtained for the concentration of radioactivity in the serum (fig. 7). The high value seen in the interval 24–25 days after oral administration of flupenthixol is explained by a high concentration in one of the two urine samples from this interval, possibly due to contamination. Roughly the same pattern as for the urinary excretion was seen for faecal excretion (table 3), but in this case the

maximum excretion in the interval 6–7 days was only seen in one of the dogs given FPT-dec., while the other three dogs showed maximum excretion at 3–4 days after injection.

Seven–37 % of the radioactivity could be extracted from the urine and 21–54 % from the faeces at an alkaline pH. The TLC of extracts of urine from dogs given FPT shows that FPT-SO was the major excretion product in the urine. In addition, FPT, HN-FPT and HN-FPT-SO were seen. The TLC extracts of faeces was to some extent interfered with by impurities, but there was indication of the presence of FPT and the sulphoxide of this compound.

TLC of urine and faeces extracts from dogs given FPT-dec. indicated that the sulphoxides of FPT and HN-FPT were the major excretion products followed by HN-FPT. The presence of FPT-dec., FPT-dec.-SO and FPT in very small amounts was also indicated.

In vitro study on hydrolysis. As shown in table 4 the amount of radioactivity extractable in the hydrolysis experiment ranged from about 100 per cent for the pure buffer to 36 per cent for the one hour sample of kidney homogenate. The relative amounts of FPT-dec. and FPT show that the highest hydrolytic activity was found in the brain homogenate and diluted blood, while the homogenates of liver, lungs and kidneys had less activity. A

Table 4.

Extraction and chromatographic analysis of *in vitro* samples.

		%-Radioactivity in DCE-phase	Distribution of radioactivity on FPT-dec. and FPT	
			%-FPT-dec.	%-FPT
Buffer	1 hour	102	82	5
	2 hours	93	77	6
Blood	1 hour	49	9	77
	2 hours	67	9	79
Brain	1 hour	72	3	70
	2 hours	90	4	85
Liver	1 hour	60	20	67
	2 hours	80	12	78
Lungs	1 hour	50	25	64
	2 hours	74	16	75
Kidneys	1 hour	36	20	64
	2 hours	55	22	67

small spontaneous degradation was observed in the buffer samples. In fig. 9 the chromatograms of the extracts of the 2 hour samples are shown. The FPT-peak shows a tendency to separate into two peaks, indicating the formation of the β-isomer of FPT. The degradation product, TFT, was seen on all the chromatograms.

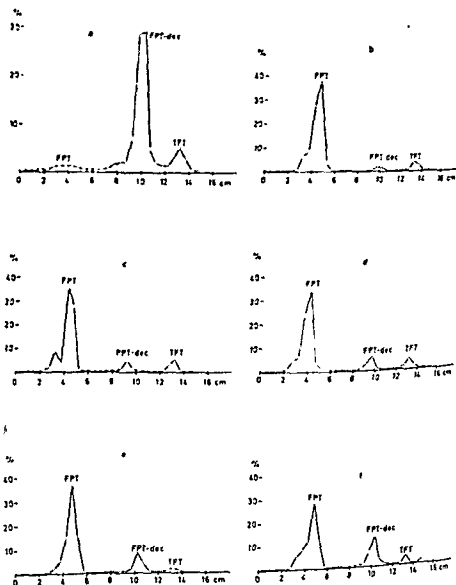


Fig. 9. Thin-layer chromatograms of extracts of 2 hour samples from the *in vitro* hydrolysis study. a: *Buffer*, b: *Brain homogenate*, c: Diluted blood, d: Liver homogenate, e: Lung homogenate, f: Kidney homogenate. Solvent system I.

Discussion

The presence of a relatively limited depot of flupenthixol decanoate at the site of injection is indicated by the large amount of radioactivity maintained in the leg, where the injection was given. The detection in the organs of unchanged flupenthixol decanoate as well as the high hydrolytic activity of the blood and the organs as demonstrated by *in vitro* experiments, suggests the release of unhydrolyzed ester from the depot and subsequent hydrolysis. Since flupenthixol is capable of passing through the blood brain barrier (JØRGENSEN *et al.* 1969), it seems unlikely that the more lipophilic flupenthixol decanoate should be unable to do so. Thus the flupenthixol found in the brain might originate from flupenthixol decanoate hydrolyzed both in the brain and elsewhere. This is not in agreement with the results on the fluphenazine ester, fluphenazine enanthate, by EBERT & HESS (1965), who suggested, that the fluphenazine found in the brain, originated solely from the ester hydrolyzed outside the brain.

The neuroleptic effect seen in rats after the administration of flupenthixol decanoate in the pharmacological studies of MØLLER NIELSEN *et al.* (unpublished) and FRANCK (1970) is presumably due to the presence of flupenthixol in the brain as this is the main substance found in brain extracts.

The distribution of radioactivity following the administration of flupenthixol decanoate to rats is identical to that found after oral administration of flupenthixol (JØRGENSEN *et al.* 1969) and very similar to that seen after the administration of other psychotropic drugs e. g. fluphenazine enanthate (EBERT & HESS 1965) and prothiadene (dosulepinum NFN) (HOREŠOVSKÝ *et al.* 1967). The substances detected in organs from dogs given unlabelled flupenthixol decanoate indicate a distribution pattern similar to that found in rats. However, when blood levels are considered, a difference is seen between rats and dogs, as rats show a maximum within the first 24 hours, while a maximum blood level is obtained in the dog 7 days after administration. The reason for this species difference is obscure, but is probably to be sought in differences in the rate of release of the drug from the depot.

A comparison between the amounts of radioactivity in blood and organs obtained after administration of flupenthixol decanoate and flupenthixol (JØRGENSEN *et al.* 1969) respectively, reveals that higher levels are maintained for a longer period of time when flupenthixol decanoate is administered, indicating that injection of the decanoic acid ester in oil causes a sustained release of drug from the depot. This is further supported by the elimination data. The elimination half-life in rats was estimated as 8 days following flupenthixol decanoate as compared to one day after flupenthixol (JØRGENSEN *et al.* 1969). Thus the biochemical findings correlate well with the prolonged effect seen in the pharmacological studies with flupenthixol decanoate (MØLLER NIELSEN

et al. unpublished; FRANCK 1970). Apart from possible traces of unchanged flupenthixol decanoate and its sulphoxide, the metabolites found in the urine and faeces are the same as those found after flupenthixol (JORGENSEN *et al.* 1969), showing that flupenthixol decanoate is largely converted to flupenthixol and that the pathway for biotransformation of flupenthixol does not change.

The presence of both isomers of flupenthixol after the administration of α-flupenthixol decanoate has been demonstrated in these studies. Since, however, the spontaneous formation of the β-isomer during the procedures has been shown, the degree of isomerisation within the organism can not be estimated.

Acknowledgements

The authors wish to thank their laboratory staff for skilful technical assistance.

REFERENCES

Bilde, Annelise & Anna Madsen: Flupenthixoldepotbehandling af schizophrene. *Nordisk Psykiatrisk Tidsskrift* 1970, XXIV, 245–249.

Ebert, A. G. & S. M. Hess. The distribution and metabolism of fluphenazine enanthate. *J. Pharmacol. Exp Therap* 1965, 148, 412–21.

Enerheim, Birgitta, C. G. Gottfries & Catherine Sundén: Kliniska försök med flupenthixol-depå-behandling vid långvariga schizofrena psykoser. *Nordisk Psykiatrisk Tidsskrift* 1970, XXIV, 239–244.

Franck, K. F.: The prolonged inhibition of the conditioned avoidance response in rats and mice after depot injection of flupenthixol decanoate in viscoleo®. Collegium Internationale Neuro-Psychopharmacologicum. VII International Congress, August 11–15, 1970, Prague, Czechoslovakia.

Gjestland, Arnulf: Flupenthixol i depotform – Nytt hjelpemiddel i psykiatrisk ettervern. *T. norske Lægeforen.* 1970, 90, 475–77.

Herberg, R. J: Determination of carbon-14 and tritium in blood and other whole tissues. *Anal Chem.* 1960, 32, 42–46.

Horešovský, O., Franc & P. Krans: Biochemistry of drug IX. The metabolic fate of a new psychotropic drug 11-(3-dimethylaminopropylidene)-6,11-dihydrodibenz-(b, e)-thiepine (prothiadene). *Biochem. Pharmacol.* 1967, 16, 2421–29.

Jørgensen, Aksel, Villy Hansen, Ulla Dahl Larsen & A. Rauf Khan: Metabolism, distribution and excretion of flupenthixol. *Acta pharmacol et toxicol.* 1969, 27, 301–13.

Remvig, Jørgen, Martin Lotz & Knud Odgaard: Flupenthixol-depot-behandling ved kronisk skizofreni. *Nordisk Psykiatrisk Tidsskrift* 1968, XXII, 392–400.

Acta pharmacol. et toxicol. 1971, 29, 359–374.

From the Department of Pharmacology, Division of Clinical Pharmacology,
Karolinska Institutet, Stockholm, Sweden

Uptake, Metabolism and Excretion of Desmethylimipramine and its Metabolites in the Isolated Perfused Rat Liver

By

Christer von Bahr and Olof Borgå

(Received October 26, 1970)

Abstract: The isolated perfused rat liver was used in order to study the metabolism of the tricyclic antidepressant drug, desmethylimipramine (DMI), which is metabolized by oxidation and subsequent conjugation. DMI labelled with tritium in a position in which it is not eliminated by metabolism, was used. Extraction procedures were developed which allowed a separation of DMI and its hydroxylated and conjugated metabolites. DMI and its metabolites were measured in perfusate plasma, bile and liver. DMI disappeared quickly from the plasma and reappeared in high concentrations in liver and bile. The drug was highly bound to proteins in perfusate plasma, while the conjugated metabolites were not bound at all. The red blood corpuscles contained approximately ten times higher concentrations of DMI than perfusate plasma but no conjugated metabolites. Only small amounts of unconjugated hydroxylated metabolites were found in perfusate plasma, liver and bile in contrast to the conjugated metabolites which occurred in considerable amounts in perfusate plasma and bile. DMI was bound to the microsomal fraction of the liver cell. The conjugated metabolites appeared mainly in the cytoplasm of the liver cell

Key-words: Desmethylimipramine – drug metabolism – perfused liver – intracellular distribution – bile excretion.

Many drugs (Brodie *et al.* 1958), aliphatic hydrocarbons (Robbins 1961) and steroid hormones (Kuntzman *et al.* 1964) are metabolized oxidatively by enzyme components in the liver endoplasmic reticulum. The oxidative step is often followed by conjugation, e. g. with glucuronic acid. Thus, polar metabolites are formed which are easily excreted in the urine and bile.

Most drug metabolism studies have been performed *in vivo* or *in vitro* with isolated liver microsomes. In the isolated perfused liver the metabolic steps can be studied simultaneously with factors closely related to metabolism, such as the uptake and transport of a drug and its metabolites in the [illegible] also the distribution of these compounds between liver, perfus[illegible] (Kalser *et al.* 1968; Nagashima [illegible] *al.* 1968).

The tricyclic antidepressant drug desmethylimipramine (DMI) is an intermediate in the main metabolic pathway of imipramine, and its metabolism has been studied in connection with pharmacokinetic investigations of the latter drug. Extensive investigations of the biotransformation of imipramine and DMI have been performed in rats and rat liver microsomes and also in man (HERRMANN & PULVER 1960; HERRMANN *et al.* 1960; BICKEL & BAGGIOLINI 1966; CRAMMER *et al.* 1968; BICKEL & WEDER 1968; CRAMMER *et al.* 1969; SJÖQVIST *et al.* 1969; CRAMMER & SCOTT 1966; OBERSTEG & BÄUMLER 1962; HERRMANN 1963). DMI is metabolized in the liver by hydroxylation, N-dealkylation and conjugation reactions. (The different metabolites are depicted in fig. 1). In man, hydroxylation occurs predominantly in the 2-position (aromatic) with a small portion reacting in the 10-position (benzylic) (CRAMMER & SCOTT 1966). In the rat only the 2-hydroxylated metabolites have been found (BICKEL & WEDER 1968).

The main purpose of this investigation was:

a) to study the time course of the disappearance of DMI in the perfused rat liver system and the concomitant appearance of its metabolites;

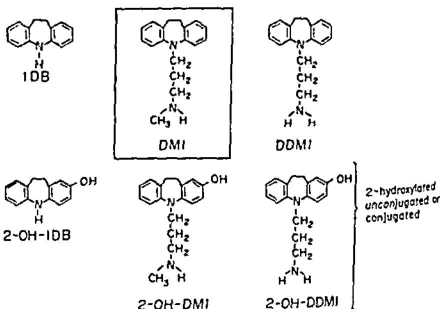


Fig. 1. Desmethylimipramine (DMI) and its main metabolites found in rats according to extensive investigations by several authors (HERRMANN *et al.* 1960; BICKEL & WEDER 1968; HERRMANN 1963). The 2-hydroxylated metabolites are conjugated mainly with glucuronic acid (HERRMANN & PULVER 1960; BICKEL & WEDER 1968).

Abbreviations: DDMI = didesmethylimipramine;
IDB = iminodibenzyl;
2-OH-DMI = 2-hydroxydesmethylimipramine.

b) to obtain information on the distribution of DMI and its metabolites in the plasma, liver and bile as well as their intracellular distribution.

Material and Methods

Animals.

Male non-starved Sprague-Dawley rats weighing 200–300 g were used as liver donors and rats weighing 500–600 g as blood donors.

In three experiments livers from male 1.5 year old homozygous GUNN rats were perfused (kindly donated by Dr. O. Hänninen, Department of Physiology, University of Turku, Turku, Finland). The plasma from these animals was dark yellow in colour (hyperbilirubinaemia).

Perfusion apparatus.

The perfusions were performed mainly according to the principles of MILLER *et al.* (1951). Our apparatus (fig. 2) was somewhat modified according to SEGLÉN & JERVELL (1969). It consists of an overflow bypass container from which the perfusate flows through a flow cuvette (Radiometer type DS 66014) containing electrodes for pH- and gas tension measurements (Radiometer, pO_2 E5046; pCO_2 E5036; Glass electrode G 265 C, Calomel electrode KS 67053). The electrodes are connected to a Radiometer Acid-Base Analyzer type PHM 71. From the flow cuvette the blood enters the liver by a piece of tubing. The liver is placed in a funnel with a cover. From this the perfusate drops down into the oxygenator, which consists of a glass tube containing a finely meshed stainless steel net for equilibration with the gas phase (94 % O_2; 6 % CO_2). The gas washing bottle, the overflow bypass container, the flow cuvette and the oxygenator are all surrounded by water jackets and thereby kept at constant temperature (37.5°). The bypass container is adjusted to give a perfusion pressure between 18–20 cm water. A thermostat is used to heat and circulate the water. All the tubing is silastic® (Dow Corning, Michigan). A roller pump is used to circulate the perfusion fluid.

Perfusion fluid.

Blood (30–50 ml) from rats anaesthetized with ether was diluted with an equal volume of Tyrode's solution and was used as the perfusion fluid. Eighty ml of the perfusion fluid contained 0.2 g dextrose and 1000 i. u. heparin. The pH of the perfusate varied between 7.4 and 7.6. The pO_2 ranged from 80–120 mmHg and pCO_2 from 10–12 mmHg on the "arterial" side of the oxygenator. The perfusate was recirculated in the system and passed through a nylon mesh filter in order to eliminate any micro-clots. The flow through the liver was 1.0–2.0 ml/g liver × min.

Preparation of the liver

The liver donor rats were anaesthetized with ether. The abdomen was opened and the bile duct located and cannulated with a polyethylene tube. Thereafter 250 [illegible]
heparin was injected into the inferior vena cava. The portal vein was ca[illegible]
the liver was cut out and placed in the funnel. The operation took 5 [illegible]
perfusion fluid left the liver through the open ends of the cut [illegible]
was perfused for 10 minutes [illegible] tus before the drug [illegible]

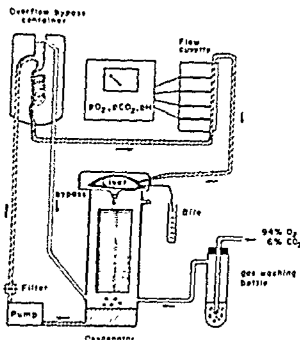


Fig. 2. Perfusion apparatus.

Perfusion experiments

The drug was administered into the overflow container. To obtain a quick distribution of the drug in the system there was a rapid bypass circulation for two minutes. Aliquots of 7 ml of perfusate were collected at five to six times.

In all the experiments fractions of the bile were sampled and its flow rate recorded. The bile flow was constant (0.28 ± 0.06 ml/hour; mean ± S. D.) during the perfusion. Only livers with continuous blood flow and bile production were used. At the end of some experiments the livers were perfused with Tyrode's solution in order to remove blood. Thereafter they were homogenized in a Potter-Elvehjem homogenizer and diluted to 5 %.

Microsomal studies.

In three experiments the liver was homogenized in 0.25 M sucrose and different liver fractions prepared according to ERNSTER *et al.* (1962). The amount of protein in the fractions was assayed according to LOWRY *et al.* (1951). In five experiments the intra- and extramicrosomal distribution of glucuronides of the 2-hydroxymetabolites of DMI was studied. For these experiments a microsomal incubation system was used (KUPFER & ORRENIUS 1970).

Binding of DMI and its metabolites in perfusate plasma and liver cell fractions.

The binding in these samples was studied by ultrafiltration as described by BORGÅ *et al.* (1969). After ultrafiltration, aliquots were taken of the ultrafiltrate and of the content of the dialysis bag and analysed for DMI and its metabolites according to extraction procedure B (see below).

Content of DMI and its metabolites in the formed elements of the blood.

After centrifugation of the perfusate, the formed elements of the blood were washed three times with Tyrode's solution followed by lysis of 0.5 ml of the formed elements in 4.5 ml of 0.2 % aqueous NH_3. DMI and its metabolites were analysed according to separation procedure B.

Chemicals.

Desmethylimipramine hydrochloride (DMI·HCl) labelled with tritium in the 10 and 11 position, with a specific activity of 13.0 mci/mmol was used (kindly supplied by AB Leo, Helsingborg, Sweden). The radiochemical purity was > 99 % as tested in three thin-layer systems (see below). The following procedure was succesfully used to remove a small amount of radioactive impurity which had the same Rf-value as iminodibenzyl (IDB). ^{3}H-DMI·HCl in water solution was acidified with acetic acid to pH 3.2 and shaken four times with hexane, which extracted the impurity but not DMI. After adjustment to pH 10.5 the DMI was extracted into ether. From the ether solution DMI was re-extracted into HCl. This acid phase was diluted to give a final solution of 2.3 mg/ml of DMI.

Thin-layer chromatography.

Thin-layer chromatography was performed on Silica Gel (Kieselgel GF_{254} nach Stahl) in the following systems:

I. Chloroform : n-propanol : sat. ammonia, 50 : 50 : 1. (Bickel & Weder 1968);
II. Chloroform : acetone : diethylamine, 5 : 4 : 1;
III. Diethyl ether : ethylene dichloride : diethylamine, 12 : 12 : 1.

Analyses of DMI and metabolites – General aspects.

The separation schemes aimed at a separation of DMI and its metabolites into fractions according to polarity and were constructed using partition coefficients as published by Weder & Bickel (1968) and Bickel & Weder (1969). These authors investigated the extractability of the majority of the imipramine metabolites in the rat over a wide pH range. By using these data, the optimum pH of the water phase and the numbers of liquid-liquid partitions for complete extraction could be calculated for each group of metabolites (bases, phenols etc.). Metabolites with a very weak basic group such as IDB and non-conjugated 2-hydroxyiminodibenzyl (2-OH-IDB) (fig. 1) appear together in one fraction by extraction at pH 3.2, while stronger bases such as DMI and didesmethylimipramine (DDMI) are not extracted at this pH. The latter bases can be separated from those containing phenolic groups, such as 2-hydroxydesmethylimipramine (2-OH-DMI) and 2-hydroxydidesmethylimipramine (2-OH-DDMI) which can be selectively re-extracted with 1 M-NaOH from a chloroform extract, leaving the non-phenolic metabolites in the chloroform phase. The conjugated hydroxylated metabolites are investigated after β-glucuronidase and arylsulphatase treatment. Each of the separated fractions contains more than one metabolite. It was not the purpose of this study to separate and estimate the individual metabolites, nor to search for new metabolites of DMI in rats, since this field has been thoroughly mapped out by other investigators.

Separation procedure A (fig. 3).

Five ml of the biological sample (plasma, 5 % liver homogenate or diluted bile) was adjusted to pH 3.2 with acetic acid and extracted with 3 × 5 ml of hexane.

The hexane layer was separated off and washed once with 10 ml of dilute acetic acid, pH 3.2, to remove any traces of DMI. The washings were discarded. The hexane phase (fraction a) contained the lipophilic weakly basic metabolites

Fig. 3. Separation scheme according to procedure A. In parenthesis is shown the anticipated (according to partition coefficients and pK_a-values) distribution of the known metabolites to the different fractions a–e.

To achieve separation of the non-conjugated and the conjugated compounds, the remaining water phase after the initial hexane extraction, was brought to pH 10 by dropwise addition of 10 M-NaOH, and the non-conjugated compounds extracted by shaking with 3 × 10 ml of chloroform. The water phase containing the conjugated metabolites was analysed separately (see below).

The phenolic metabolites in the combined chloroform layers were separated from the non-phenolic metabolites by shaking the chloroform twice with 10 ml of 1 M-NaOH. The chloroform layer (fraction b) containing the non-phenolic bases DMI and DDMI was evaporated before the measurement of radioactivity.

The alkaline water phase was adjusted to pH 10 with 9 M-HCl. The phenolic bases (2-OH-DMI and 2-OH-DDMI) corresponding to the non-conjugated fraction of these metabolites in the sample, were extracted with 3 × 7 ml of chloroform. The chloroform layers were combined, and the radioactivity of this fraction (c) was measured.

The aqueous phase containing the conjugated metabolites was adjusted to pH 5.5 with 1 M-HCl. 0.1 ml of "Glusulase Boehringer" containing β-glucuronidase and arylsulphatase was then added and the incubation was performed at 37° for 16 hours.

The aqueous phase was adjusted to pH 10 with 10 M-NaOH and shaken with 3 × 10 ml of chloroform. The chloroform layer (fraction d) containing 2-OH-DMI, 2-OH-DDMI and 2-OH-IDB was then separated from the aqueous phase (fraction e), which still contained considerable amounts of radioactivity (see table 2). Part of this activity may have been due to incomplete splitting of the conjugates, but attempts to achieve a more complete hydrolysis by repeated enzymatic treatment or acid hydrolysis were unsuccessful. The nature of the remaining polar metabolites in the aqueous phase was not further investigated.

It must be pointed out that we use the radioactivity in fraction b as an estimate of DMI (parent drug) although it is known that the metabolite DDMI also appears in this fraction. However, the interference of DDMI may for practical purposes be neglected since DDMI is found only in small amounts in rat tissues and excreta (BICKEL & WEDER 1968).

Separation procedure B.

The sample (1–5 ml) was adjusted to pH > 12 and extracted with 3 × 5 ml of hexane. The combined hexane layers contain DMI together with the minor metabolites DDMI and IDB.

The aqueous phase was adjusted to pH 10 and extracted with 2 × 10 ml of chloroform. The chloroform phase contains the unconjugated 2-hydroxylated metabolites. The remaining water phase is then further analysed according to procedure A.

The radioactivity in the hexane extract is used as an estimate of DMI concentration and the minor metabolites DDMI and IDB which are present in this extract, are neglected.

Recoveries.

Since the partition coefficient for DMI between chloroform and water is so high (> 99 : 1) (WEDER & BICKEL 1968), the recovery of DMI in procedure A is considered to be quantitative. Although the extractability of DMI in hexane is somewhat less (WEDER & BICKEL 1968), the recovery in procedure B is already 98.6 ± 1.1 % (mean ± S.D.) after the first two of the three hexane extraction steps. Since IDB is more lipophilic than DMI, three extractions with hexane is considered to give a quantitative recovery of this metabolite (procedure A).

The recovery of the more polar phenolic bases (e. g. 2-OH-DMI) was estimated by repeated extractions of authentic plasma samples with chloroform (procedures A and B). The recovery was better than 99 %.

Radioactive measurements.

Determinations of radioactivity present in the hexane phase (fraction a) were performed in a toluene scintillation mixture (4 g of PPO and 0.5 g of dimethyl-POPOP in 1 litre of toluene). Chloroform phases (fractions b, c and d) were evaporated to dryness in glass counting vials (plastic vials are not suitable since the plastic wall can absorb lipophilic compounds during the evaporation procedure) before the addition of the scintillator. Aqueous phases were counted in Maickels mixture (BEAVEN & MAICKEL 1964). Counting efficiencies in each sample were determined by the internal standard method.

Results

DMI and its metabolites in perfusate medium.

The time course of the disappearance of DMI and appearance of its metabolites in perfusate plasma is shown in fig. 4. DMI disappeared quickly from the perfusate plasma (half-life 4–9 min.; n = 5) with a concomitant appearance of conjugated metabolites. The total radioactivity showed an initial decrease followed by an increase.

In the perfusate plasma taken at a later period from a perfusion experiment, 83 % of the DMI (at a concentration of 0.8 μM) was bound to plasma proteins whereas no binding of the conjugated metabolites (concentration 6.0 μM) could be detected. Red blood corpuscles (RBC) contained higher concentrations of DMI than the perfusion plasma, the ratio being approximately constant with time (table 1). No conjugated metabolites were detected in the RBC.

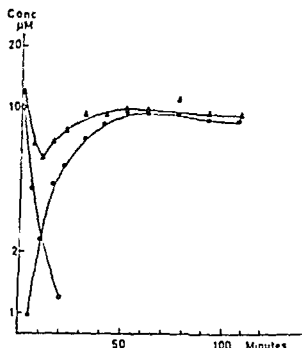


Fig. 4. Disappearance of DMI and appearance of its polar metabolites in perfusate plasma analysed according to procedure II. △——△ total radioactivity before extraction, ○——○ DMI (hexane extract), ●——● total polar metabolites (water phase after hexane extraction). Dose of DMI: 2.5 mg/100 ml perfusate.

Distribution between perfusate, bile and liver.

Table 2 shows the concentration of DMI and its metabolites in perfusate plasma, bile and liver. DMI was found in high concentrations in the bile and liver. Only very small amounts of unconjugated hydroxylated metabolites were found in the plasma, bile and liver. The conjugated metabolites, however, were found in considerable concentrations in the plasma and especially in the bile.

Table 1.

DMI concentration in perfusate plasma and red blood corpuscles at different times in a perfusion experiment

Time (minutes)	(x) DMI in red blood corpuscles (μM)	(y) DMI in perfusate plasma (μM)	Ratio $\frac{x}{y}$
0	65.0	6.2	10.5
15	14.0	1.1	12.7
45	4.2	0.56	7.5

Table 2.

Concentration (μM) and relative abundance in per cent of totally recovered compounds. The letters for the different metabolic fractions are given in fig. 3. Dose of DMI: 3.1 mg/100 ml perfusate. Data from one of two experiments with similar results.

	Time-point (min.)	a Weak bases	b Strong bases	c Unconjugated phenolic bases	d Conjugated phenolic bases	e Unidentified polar metabolites
Plasma	1.5	0.06 0.2 %	23.9 91.5 %	0.02 0.1 %	1.8 6.8 %	0.36 1.4 %
	13	0.28 2.4 %	8.2 70.5 %	0 02 0.2 %	2.0 16.8 %	1.2 10.1 %
	29	0.31 2.2 %	5.3 38.9 %	0.04 0.3 %	6.3 46.7 %	1.6 11.9 %
	44	0.26 1.5 %	3.6 20.3 %	0 05 0.3 %	10.9 62.3 %	2.7 15.6 %
	70	0.20 1.1 %	2 9 15.6 %	0.07 0.4 %	12.8 68 %	2.8 14.9 %
Bile	11.5	8 1.6 %	70 14.2 %	2 0.4 %	350 71.1 %	63 12.8 %
	28	21 1.2 %	250 11.1 %	7 0 4 %	1300 73.6 %	240 13.7 %
	44	45 1.3 %	270 7.7 %	12 0.3 %	2720 77.9 %	450 12.8 %
	70	25 1.1 %	150 6.8 %	9 0 4 %	1670 75.6 %	360 16.1 %
Liver*	70	29 13 8 %	160 78.2 %	0.3 0.1 %	9.1 4.4 %	7.3 3.5 %

* Concentrations in the liver are given in μmol/1000 g.

For DMI and IDB high concentration ratios were found between the bile and perfusate plasma (table 3), whereas the ratios between the bile and liver were close to unity. In contrast the conjugated metabolites showed a high concentration ratio between bile and liver and approximately a 1 : 1 ratio between plasma and liver.

Subcellular distribution of DMI and its polar metabolites.

Some livers were perfused with DMI and at the end of the perfusions different liver cell fractions were prepared. DMI was found in considerable

Table 3.

Concentration ratios at the end of the perfusion (70 min.) for DMI and its metabolites between bile, liver and perfusate plasma. The values are means of two experiments.

Type of conc. ratio	DMI	Weak bases (IDB)	Conjugated phenolic bases
Bile/plasma	70	100	190
Bile/liver	0.75	0.70	130
Liver/plasma	100	150	1.4

amounts in the whole homogenate, 12,500 g pellet, 105,000 g supernatant and in the microsomal fraction. When 12,500 g supernatant fractions obtained from homogenized perfused livers were centrifuged at 105,000 g (50 min.) the polar metabolites were found mainly in the supernatant fraction. The ratio between the amounts in the 105,000 g supernatant and microsomal fraction was more than ten times higher for conjugated metabolites than for the parent drug.

The *in vitro* "binding" of DMI to the microsomes and the 105,000 g supernatant fraction was also measured by ultrafiltration. DMI was considerably "bound" to the microsomes (table 4).

The subcellular distribution of glucuronides formed from DMI by hydroxylation and subsequent glucuronidation, was also studied with another technique. DMI was incubated *in vitro* with microsomes in the presence of UDPGA. After incubation the microsomes were centrifuged down at 105,000 × g for 90 minutes. The appearance of the metabolites (glucuronides) was estimated in the supernatant and in the corresponding microsomal fraction. Most of the metabolites were found in the supernatant (fig. 5).

Table 4.

Binding of DMI to different cell fractions. DMI was added *in vitro* to the fractions, which were diluted with 0.5 M Tris buffer pH 7.5 to give the same protein concentration. The binding was measured by ultrafiltration through dialysis membranes with the same method as used for plasma protein binding (BORGÅ *et al.* 1969). The values are the mean of two experiments.

Cell fraction	% Bound DMI	Protein conc. (mg/ml)	DMI conc. (μM)
105,000 g supernatant	16	2	80
Microsomal pellet (resuspended)	67	2	80

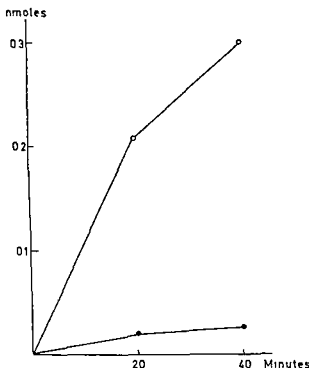


Fig. 5. Distribution of glucuronides (formed from hydroxylated metabolites) between microsomal pellet and the corresponding 105,000 g supernatant. DMI was added to give an initial concentration of 3×10^{-6} M and UDPGA to give 4.5×10^{-5} M. Amount of glucuronides in the microsomal pellet (●——●) and in the 105,000 g supernatant (○——○).

Fate of DMI in the perfused liver under different experimental conditions.

Since it is known that the plasma disappearance of some drugs is dose dependent (Dayton *et al.* 1967; Levy 1968), some livers were perfused with higher doses of DMI (30–74 mg/60 ml perfusate; n = 4). This should give concentrations of DMI in whole blood ranging from about 500–1200 µg/ml. The plasma concentrations were however considerably lower as found in the other experiments, due to the distribution of DMI to the red blood corpuscles. At these doses the drug disappeared from the perfusate plasma with similar initial half-lives (5–8 min.) as in the controls. However, the livers were macroscopically affected and the amount of metabolites in the perfusate was reduced. Their concentration after 20 min. of perfusion was less than one per cent of the initial DMI concentration. The blood flow through the livers decreased, the livers became swollen and the bile production diminished. In one experiment (74 mg/60 ml perfusate) with marked haemolysis, the bile was clear red in colour. All these livers showed good perfusion and showed good bile production before the administration of the drug.

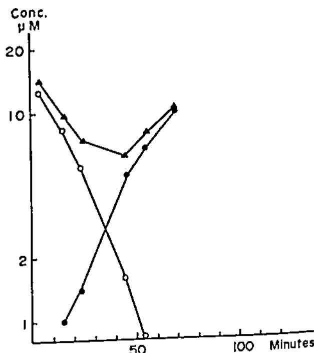


Fig. 6. Perfusion of DMI in liver from GUNN rat. Disappearance of DMI and appearance of its polar metabolites in perfusate plasma analysed according to procedure B. ▲——▲ total radioactivity before extraction, ○——○ DMI (hexane extract), ●——● total polar metabolites (water phase after hexane extraction). Dose of DMI 2.5 mg/85 ml perfusate.

The capacity of homozygous GUNN rats to conjugate bilirubin with glucuronic acid is poor (LESTER & SCHMID 1964). However, on perfusion of DMI similar results were obtained as in normal rats and conjugated metabolites were formed (fig. 6). The half-lives of DMI in the perfusate plasma were 9, 10 and 12 minutes. On incubation of DMI in microsomes from these rats (in the presence of UDPGA) glucuronides were also formed (fig. 7).

Discussion

The isolated perfused rat liver was used to study the metabolism of the tricyclic antidepressant drug desmethylimipramine, which is metabolized by oxidation and subsequent conjugation. This preparation was chosen for the following reasons:

a) It eliminates or minimizes *in vivo* factors such as absorption from the intestinal tract, tissue distribution and renal excretion.

b) The metabolite pattern probably reflects the *in vivo* situation better than that produced with other *in vitro* techniques.

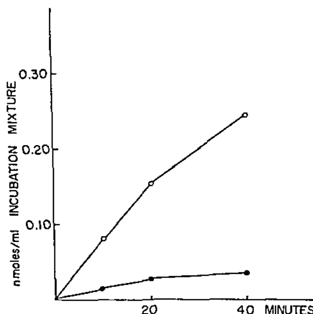


Fig. 7. Formation of glucuronides (formed from hydroxylated metabolites) following incubation of DMI (0.9 × 10^{-5} M) in isolated liver microsomes (GUNN rat). ○——○ = in the presence of UDPGA (2 × 10^{-4} M) ●——● without UDPGA.

In all the experiments there was an initial decrease of total radioactivity in the perfusate plasma, reflecting a net uptake in the liver. After a short lag period the radioactivity increased again, mainly due to appearance of polar metabolites which were less bound to the liver. DMI disappeared quickly from the perfusate plasma. The results with the higher doses of DMI show that plasma disappearance of a drug may not only reflect metabolism rate but also to a great extent, tissue uptake.

The disappearance of DMI with time from the red blood corpuscles followed approximately that in perfusate plasma. This indicates a rapid equilibration between the two compartments

DMI was bound about 80 % to proteins in perfusate plasma, but the binding of the conjugated metabolites was negligible. The polarity of these metabolites and the minute binding to plasma proteins are favourable factors for renal elimination *in vivo*.

The metabolism of a compound is closely related to other functions such as liver uptake (SMITH 1966), distribution and binding in the liver cell and excretion into perfusate and bile of the parent drug and its metabolites. DMI was found in high concentrations in the liver and bile. The concentration of the drug in the bile was considerably higher than in the perf[illegible] pl[illegible]

but it was similar (at a late time point) to that in the liver (table 3), which indicate that DMI is concentrated in the liver. Such an uptake has been described by several investigators (SMITH 1966; LEVI *et al.* 1968). In contrast to DMI the conjugated metabolites showed a high concentration ratio between bile and liver. Active transport of glucuronides into the bile and a possible relationship between metabolism (glucuronidation) and bile excretion have been discussed (SMITH 1966; LEVINE *et al.* 1970). The mechanism(s) by which a drug and its metabolites reach the bile is however still unknown.

After the uptake in the liver DMI is distributed to and metabolized in the endoplasmic reticulum, to which cell fraction the drug is markedly bound (table 4) (DINGELL *et al.* 1961). The conjugated metabolites appeared mainly in the cytoplasm, which is in agreement with the findings of DEMISCH *et al.* (1969), who studied the subcellular distribution of testosterone and its metabolites after liver perfusions. Our findings indicate that the metabolites formed may leave the liver cell through the cytoplasm and unlike e.g. albumin through the lumen of the endoplasmic reticulum. In the distribution experiments with the liver microsomes (fig. 5) the unimpaired condition of the microsomal vesicles during the incubation and subsequent centrifugation, and the permeability of the vesicles for the conjugated metabolites are critical. If such metabolites easily pass through the microsomal membrane, their actual distribution may be difficult to determine with this technique.

It was interesting to find that the homozygous GUNN rats formed glucuronides, since they show defective glucuronidation of bilirubin. Our findings may indicate that different glucuronyltransferases are involved in the glucuronidation of various substrates. However, other explanations are also possible. The method of studying the reaction and the polarity of the substrates may influence the results. In our case a lipophilic drug which must be hydroxylated before glucuronidation was used.

In liver microsomal preparations only oxidated unconjugated metabolites of DMI occur, unless UDPGA is added to the system (VON BAHR 1970a). This accumulation can cause product inhibition (VON BAHR 1970; VON BAHR & ORRENIUS 1970, unpublished results.) In our perfusion experiments the situation was different. Only very small amounts of unconjugated hydroxylated metabolites were found in the liver, perfusate plasma and bile (table 2), in contrast to the conjugated metabolites which were found in appreciable amounts in the perfusate plasma and bile (VON BAHR 1970b). These findings suggest that a large fraction of the hydroxylated metabolites is conjugated quickly after the hydroxylation and possibly without leaving the liver cell. The conjugates are then rapidly excreted. Hence the possible role of product inhibition of the metabolism caused by accumulation in the liver of unconjugated hydroxylated metabolites may be diminished by conjugation and excretion.

The overall studies of the pharmacokinetics and metabolism of DMI in the perfused rat liver presented in this paper give a deeper understanding of the problems involved in extrapolating drug metabolic data obtained *in vitro* to the situation *in vivo*.

Acknowledgements

The authors would like to thank Miss Ingegärd Johansson for skilful technical assistance.

This study was supported by grants from the Swedish Medical Research Council (B70-21x-1021-05 and B70-13x-2471-03), the National Institutes of Health, Bethesda, Maryland, U.S.A. (Gm 13978-05) and by funds from the Karolinska Institutet and Svenska Sällskapet för Medicinsk Forskning.

REFERENCES

von Bahr, C.: Hydroxylation and subsequent glucuronidation of desmethylimipramine in isolated perfused liver and liver microsomal preparations from the rat. *J. Clin. Invest.* 1970a, 25, Suppl. 113, 15–15.

von Bahr, C.: Binding to cytochrome P-450 and metabolism of desmethylimipramine in rat liver microsomes. *Acta pharmacol. et toxicol.* 1970b, 28, Suppl. 1, 13–13.

Beaven, M. A. & R. P. Maickel: Stereoselectivity of norepinephrine storage sites in heart. *Biochem. Biophys. Res. Commun.* 1964, 14, 509–513.

Bickel, M. H. & M. Baggiolini: The metabolism of imipramine and its metabolites by rat liver microsomes. *Biochem. Pharmacol* 1966, 15, 1155–1169.

Bickel, M. H. & H. J. Weder: The total fate of a drug: Kinetics of distribution, excretion, and formation of 14 metabolites in rats treated with imipramine. *Arch. int. Pharmacodyn.* 1968, 173, 433–463.

Bickel, M. H. & H. J. Weder: Buccal absorption and other properties of pharmacokinetic importance of imipramine and its metabolites. *J. Pharm. Pharmacol.* 1969, 21, 160–168.

Borgå, O., D. L. Azarnoff, G. Plym Forshell & F. Sjöqvist: Plasma protein binding of tricyclic antidepressants in man. *Biochem Pharmacol.* 1969, 18, 2135–2143.

Brodie, B. B., J. R. Gillette & N. B La Du: Enzymic metabolism of drugs and other foreign compounds. *Ann. Rev. Biochem* 1958, 27, 427–454.

Crammer, J. L. & B. Scott: New metabolites of imipramine. *Psychopharmacologia* (Berl.) 1966, 8, 461–468.

Crammer, J. L., B. Scott, H. Woods & B. Rolfe: Metabolism of ^{14}C-imipramine. I. Excretion in the rat and in man. *Psychopharmacologia* (Berl.) 1968, 12, 263–277.

Crammer, J. L., B. Scott & B. Rolfe: Metabolism of ^{14}C-imipramine: II: Urinary metabolites in man. *Psychopharmacologia* (Berl.) 1969, 15, 207–225.

Dayton, P. G., S. A. Cucinell, M. Weiss & J. M. Perel: Dose-dependence of drug plasma level decline in dogs. *J. Pharmacol. exp. Ther.* 1967, 158, 305–316.

Demisch, K., P. Birkelbach, U. Ammedick & W. Staib: Subcellular distribution of testosterone metabolites after perfusion of isolated livers of guinea pigs. *Acta Endocrin.* 1969, 62, 147–152.

Dingell, J. V., W. A. M. Duncan & J. R. Gilette: Studies on the binding of and chlorpromazine in various tissues. *Fed. Proc.* 1961, 20, 173c–173c.

Ernster, L., P. Siekevitz & G. E. Palade: Enzyme-structure relationships in the endoplasmic reticulum of rat liver. A morphological and biochemical study. *J. Cell. Biol.* 1962, 15, 541–562.

Herrmann, B. & R. Pulver: Der Stoffwechsel des Psychopharmakons Tofranil. *Arch. int Pharmacodyn.* 1960, 126, 454–469.

Herrmann, B., W. Schindler & R. Pulver: *Papier-Chromatographischer Nachweis von Stoffwechselprodukten des Tofranil. Med. exp.* 1960, 1, 381–385.

Herrmann, B.: Quantitative Methoden zur Untersuchung des Stoffwechsels von Tofranil *Helv. physiol. pharmacol. Acta.* 1963, 21, 402–408.

Kalser, S. C., E. J. Kelvington & M. M. Randolph: Drug metabolism in hypothermia. Uptake, metabolism and excretion of S^{35}-sulfanilamide by the isolated perfused rat liver. *J. Pharmacol. exp. Ther.* 1968, 159, 389–398.

Kuntzman, R., M. Jacobson, K. Schneidman & A. H. Conney: *Similarities between oxidative drug metabolizing enzymes and steroid hydroxylases in liver microsomes. J. Pharmacol. exp. Ther.* 1964, 146, 280–285.

Kupfer, D. & S. Orrenius: Interaction of drugs, steroids and fatty acids with liver microsomal cytochrome P-450. *Europ. J. Biochem.* 1970, 14, 317–322.

Lester, R. & R. Schmid: Bilirubin metabolism. *New. Engl J. Med.* 1964, 270, 779–786.

Levi, A. J., Z. Gatmaitan & I. M. Arias: The cytoplasmic proteins from rat liver and their role in hepatic uptake of sulfobromophtalein (BSP) and bilirubin. *J. Clin Invest.* 1968, 47, 61a–61a.

Levine, W. G., P. Millburn, R. L. Smith & R. T. Williams: The role of the hepatic *endoplasmic reticulum in the biliary excretion of foreign* compounds by the rat. *The effect of phenobarbitone and SKF 525–A (Diethylaminoethyl diphenylpropylacetate). Biochem. Pharmacol.* 1970, 19, 235–244.

Levy, G. H.: Dose dependent effects in pharmacokinetics. In: *Importance of fundamental principles in drug evaluation.* (Ed. D. H. Tedeschi & R. E. Tedeschi). Raven Press, New York 1968, 141–172.

Lowry, O. H., N. J. Rosebrough, A. L. Farr & R. J. Randal: Protein measurement with the folin phenol reagent. *J. Biol. Chem* 1951, 193, 265–275.

Miller, L. L., G. G. Bly, M. Watson & W. F. Bale: The dominant role of the liver in plasma protein synthesis. *J. exp. Med.* 1951, 94, 431–453.

Nagashima, R., G. Levy & E. J. Sarcione: Comparative pharmacokinetics of coumarin anticoagulants III. Factors affecting the distribution and elimination of bishydroxy-*coumarin (BHC) in isolated perfusion studies. J. Pharm. Sci.* 1968, 57, 1881–1888

Obersteg, J. I & J. Bäumler: *Suicid mit dem Psychopharmakon Tofranil. Arch. Toxicol* 1962, 19, 339–344.

Robbins, V C.: Enzymatic omega oxidation of fatty acids. *Fed. Proc.* 1961, 20, 272–272.

Seglén, P. O. & K. F. Jervell: A simple perfusion technique applied to glucocorticoid regulation of tryptophan oxygenase turnover and bile production in the isolated rat liver. *Hoppe-Seylers Z. physiol. Chem.* 1969, 350, 308–316.

Smith, R. L.: The biliary excretion and enterohepatic circulation of drugs and other organic compounds. *Arzneimittelforsch.* 1966, 9, 299–360.

Sjöqvist, F., W. Hammer, O. Borgå & D. L. Azarnoff: Pharmacological significance of the plasma level of monomethylated tricyclic antidepressants. In: *The present status of psychotropic drugs.* Proc. Coll. Internat. Neuropsychopharmacol. Tarragona, Spain, April 1968. Excerpta Med. Internat. Congr. Ser. No. 180, 1969, 128–136.

Weder, H. J. & M. H. Bickel: Separation and determination of imipramine and its metabolites from biological samples by gas-liquid chromatography. *J. Chromatog.* 1968, 37, 181–189.

Acta pharmacol. et toxicol. 1971, 29, 375–384.

From the Institute of Occupational Health, Oslo, Norway

Biotransformation of Methyl Mercuric Salts in the Mouse Studied by Specific Determination of Inorganic Mercury

By

Tor Norseth

(Received September 25, 1970)

Abstract: Biotransformation of methyl mercuric chloride in the mouse was followed by the specific determination of inorganic mercury after a single injection of the organomercurial. Inorganic mercury was detected in the blood, brain, liver, kidney, spleen and in the intestinal cells. Inorganic mercury was also found in the bile and faeces. The results indicate a role of biotransformation in the excretion of mercury after the injection of methyl mercuric salts. Inorganic mercury was preferentially excreted in the faeces. Differences in pharmacokinetics of methyl mercuric salts between rats and mice have been discussed with regard to differences in biliary excretion and enterohepatic circulation. The importance of taking into account species differences in the evaluation of toxic hazards of methyl mercuric salts has thus been demonstrated.

Key-words: Methyl mercuric chloride – biotransformation – species differences.

Biotransformation of methyl mercuric salts may lead to a cleavage of the carbon-mercury bond releasing mercury, hereafter called inorganic mercury. This reaction was recently studied in the rat by specific determination of inorganic mercury in various organs after intravenous injection of methyl mercuric chloride (NORSETH & CLARKSON 1970a and b). Biotransformation was found to be of importance for both the elimination and distribution of mercury. There are differences in the organ distribution and excretion of methyl mercuric salts in rats and mice (BERGLUND & BERLIN 1969), and this may be related to the biotransformation reaction. Species differences are of extreme importance for the evaluation of hazards in man based on animal experiments. This paper therefore presents a study on the biotransformation of methyl mercuric salts in the mouse by the methods previously used for rats.

Materials and Methods

Female mice weighing about 25 g were injected intravenously with 25 labelled mercury as methyl mercuric chloride (1 mg Hg/kg). The was prepared by exchange between mercuric chloride labelled with

mercuric chloride (CROSS & PINEAN 1951). The method was modified as described in a previous paper in order to obtain labelled methyl mercury with less than 0.5 % of inorganic mercury (NORSETH & CLARKSON 1970a). Inorganic mercury was analysed by a modification of the isotope exchange method (CLARKSON *et al.* 1965). The method is based on the principle that aqueous solutions of mercuric mercury are capable of undergoing exchange with mercury vapour in contact with the solution. Mercury bound covalently to a carbon atom does not undergo exchange, or does so to a very limited extent. Thus by using a microdiffusion chamber with unlabelled metallic mercury in one compartment, and the sample to be analyzed containing the mixture of methyl mercury and mercuric chloride both labelled with ^{203}Hg in the other, the relative amount of inorganic mercury can be determined. Since the mercury pellet contains about 1.3 g of mercury (0.1 ml), and the total amount of mercury in the other compartment never exceeds 2 µg, at isotopic equilibrium practically all the labelled inorganic mercury from the solution is recovered in the pellet. The detailed use of the method has been described previously (NORSETH & CLARKSON 1970a). The same correction

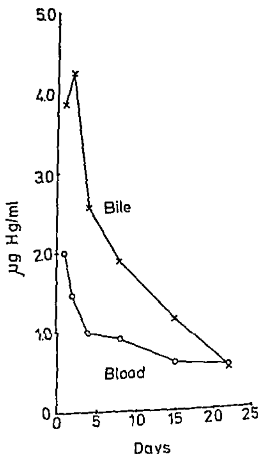


Fig. 1. Total mercury in blood and in bile. Bile was pooled from 6 gall bladders for each time interval. Blood from 3 mice was pooled and the values represent the mean of two determinations on each of two pools per time interval.

factors and methods of calculation were used. The correction factor for the scintillation counter was determined specifically as this factor is related to the spectrometer settings of the counter. The self-absorption factor for the mercury pellet was found to be 2.30, and this was used throughout this work.

Bile was collected from the gall bladder of 5–10 mice at the time intervals indicated. Samples of organs and body fluids were otherwise treated as described previously (NORSETH & CLARKSON 1970a). Procedures for thin-layer chromatography and electrophoresis of bile containing mercury compounds were as previously described (NORSETH & CLARKSON 1970c).

Results

The concentration of mercury in the blood one day after the injection of 25 µg of mercury as methyl mercuric chloride was about 2 µg/ml (fig. 1). After 22 days, the longest time interval tested, the concentration had fallen to 0.6 µg/ml. This is about half the concentration of mercury in the bile at all

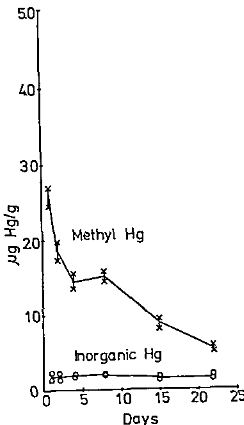


Fig. 2. Methyl mercury and inorganic mercury in the liver. Each value represents one determination on three organs pooled.

Table 1.

Inorganic mercury in blood and bile.

	Time (days)					
	1	2	4	8	15	22
Blood[a]	4.7	3.8	4.0	1.6	3.5	2.6
Bile[b]	1.8	0.8	3.2	4.4	7.1	5.2

Values are given as per cent of total per ml.

[a] Mean of 2 determinations on 2 pools of blood from 3 mice each.

[b] Bile from 6 mice pooled.

time intervals, except after 22 days. At this time, the amount of mercury in the bile and blood was approximately the same (fig. 1).

The concentration of mercury in the liver was higher than in the blood at all time intervals, but lower than in the bile (fig. 2). The liver content of organic mercury decreased at approximately the same rate as mercury in the blood, but the amount of inorganic mercury in the liver did not change significantly with time after injection (fig. 2). About 0.2 µg/g of inorganic

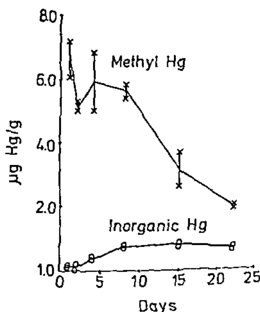


Fig. 3. Methyl mercury and inorganic mercury in the kidney. Each value represent one determination on three organs pooled.

Table 2.

Total and inorganic mercury in organs.

Days	Spleen		Colon		Small intestine		Brain	
	Total µg/g	Inorganic %	Total µg/g	Inorganic %	Total µg/g	Inorganic %	Total µg/g	Inorganic %
1	1.49	3.5	0.67	11.0	1.48	4.5	0.41	1.9
	1.34	4.1	0.82	7.2	1.37	3.9	0.52	2.0
2	1.12	4.7	0.51	10.0	1.01	1.8	0.45	1.9
	1.39	4.0	0.54	10.2	0.76	2 3	0.54	3.7
4	1.23	11.4	0.54	21.4	1 04	4.0	0.63	2.0
	0.79	5.5	0.46	21.1	0.77	3.1	0.43	6.0
8	0.86	6.8	0.47	22.0	0.74	2.1	0.61	3.5
	0.76	7.6	0.52	27.6	0.88	4.5	0.64	4.6
15	0.69	10.7	0.34	22.0	0.61	3.8	0.61	14 0
	0.74	6.6	0.29	24.1	0.58	4.0	0.62	7.0
22	0.27	13.5	0.16	–	0.40	5.8	0.35	10.0
	0.29	4.4	0.14	31.9	0.31	6.2	0.39	5.4

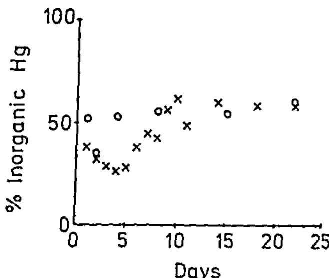


Fig. 4. Inorganic mercury in faeces and intestinal contents. Values are given as per cent of total mercury in the sample. Circles represent faecal content, other values are from excreted faeces.

mercury was found in the liver throughout the time period tested. The relative amount of inorganic mercury in this organ thus increased with time, and both the relative and absolute amount of inorganic mercury was higher in the liver than in both the blood and bile at all time intervals (*table 1*). Therefore, there may be a release of inorganic mercury from the liver to the bile where it is excreted, and to the blood where it is redistributed to other organs.

The kidney contained the highest concentration of mercury of all the organs tested (fig. 3). The rapid fall in mercury content already seen on day 2 in the blood and in the liver was not found in the kidney. The initial concentration of about 6 µg/g did not change during the first week, while the relative amount of inorganic mercury, increased. After about one week the content of organic mercury fell rapidly, while the inorganic mercury stabilized at about 0.4 µg/g, the highest value being found after 15 days (fig. 3). The relative amount of inorganic mercury was not higher than in the liver, but the time course of relative inorganic mercury accumulation was different. In the liver, there was a rapid increase followed by almost constant values for the time period tested compared to the kidney which had a slow increase over 15 days (figs. 2 and 3).

The mercury content in the brain was low as compared to the liver and kidney, i.e. 0.47 µg/g on day one, increasing to 0.62 µg/g on day 15, and falling to about half that value after 22 days (table 2). The time course of mercury uptake was thus different from both the liver and kidney. The brain

contained from 1.9 % to 14.0 % of inorganic mercury. The increase with time of the relative amount of inorganic mercury in the brain is too small for final conclusions and needs further confirmation.

The spleen contained slightly less mercury than the blood, 1.41 μg/g on day one, falling to 0.28 μg/g after 22 days. The relative amount of inorganic mercury was from 3.5 % to 13.5 %, or about the same as in the blood, demonstrating the close relationship between these two organs (table 2).

Cells from the small intestine contained twice as much mercury as cells from the colon, 1.43 μg/g and 0.70 μg/g respectively after one day (table 2). The same relative difference was found throughout the time period investigated. The intestinal content of mercury may thus influence cellular concentration. The bile deposits an unknown daily amount of mercury in the small intestine. Some of this mercury is probably reabsorbed. Cells from the colon, on the other hand, may take up inorganic mercury from the intestinal content. The relative amount of inorganic mercury in these cells was 9.1 % after one day, increasing to 31.9 % on day 22, as compared to 1.8 % and 6.2 % in the small intestinal cells for the same time interval (table 2). The faeces contained from about 25 % to 60 % inorganic mercury during the time period tested. This supports the assumption of mercury uptake, but does not necessarily mean that reabsorption occurs (fig. 4). Based on the present results, the excretion of mercury in the colon cannot be excluded (BERLIN & ULLBERG 1963a and b). Specific excretion of inorganic mercury is unlikely as the relative amount of inorganic mercury in the faeces was lower or equal to that

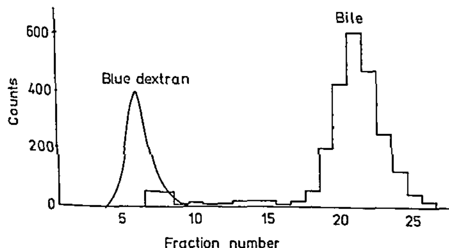


Fig. 5. Sephadex G-200 chromatography of bile in 0.1 M phosphate buffer pH 8.0 containing 1.0 N sodium chloride and 0.02 % sodium azide. Blue dextran was added as a marker and recorded by light absorption as indicated. Fractions of 2.2 ml were collected and counted as shown.

Table 3.

Thin-layer chromatography and electrophoresis of standards and bile samples.

Sample	Rf value TLC System I	Rf value TLC System II	Electrophoresis + movement (cm)
CH_3Hg-Cl	0.1	0	0-1
CH_3Hg-cysteine	0.8	0.4	1-2
CH_3Hg-glutathione	0.7	0.4	5
Bile	0.8*	0.4*	5

* A small amount remained at the starting point.
System I: Ethanol 96 %/NH_4OH 25 % – 70/30
System II: Propanol/water – 70/30
Electrophoresis: Barbiturate buffer pH 8.6 (Beckman B-2)
All standards were added to normal bile before running.

in the coecum. Taking the low relative amount of inorganic mercury and high concentration of total mercury in the bile into consideration, the relative amount of inorganic mercury in the small intestine must be lower than in colon.

In order to compare the present results with those for rats, the bile from mice was studied by thin-layer and column chromatography, and also by electrophoresis. The major part of the mercury in the bile is excreted in the form of small molecular compounds (fig. 5). Based on the Rf values in the two thin-layer chromatography systems, it is possible that this compound is methyl mercuric cysteine as found in the rat (table 3). Electrophoresis showed, however, that the mercury compounds in bile from mice and rats are not identical. A small amount, less than 10 % of the total, of the mercury found in mouse bile moved according to the methyl mercuric cysteine marker while the greater part moved in the same way as methyl mercuric glutathione.

Discussion

Biotransformation of methyl mercuric salts with release of inorganic mercury, meaning mercury not bound covalently to a carbon atom, takes place in the mouse, as well as in the rat (Norseth & Clarkson 1970b).

An initial release of inorganic mercury from methyl mercury in the mouse was found by Østlund (1969), but he could not find any inorganic mercury in significant amounts in any organ, based on the extraction methods for the intact organomercurial for time periods up to 4 hours.

By specifically analyzing inorganic mercury after exposure to methyl mercuric chloride, inorganic mercury has been demonstrated in all the organs tested.

The liver and kidney contain the highest relative amounts of inorganic mercury among the organs, but as much as about 50 % of the total faecal excretion of mercury during the first 22 days after a single injection of methyl mercuric chloride was found to be inorganic. Nevertheless, the biotransformation of releasing inorganic mercury is a slow reaction, much slower than for phenyl mercuric salts (MILLER *et al.* 1960; GAGE 1964; NORSETH & CLARKSON 1970b), though a definite amount of inorganic mercury is formed. In addition the biotransformation also seems to be of importance for the excretion of mercury in mice, as previously described for the rat (NORSETH & CLARKSON 1970c).

With a biological half-life of 8 days for a dose of 1 mg Hg/kg (ØSTLUND 1969), 30 to 40 % of the injected dose may have undergone biotransformation during the first 20 days. The degree of biotransformation may be dose-dependent if enzymatic processes are involved, and may thus offer an explanation for the dose-dependent half-life reported previously. The nature of the biotransformation reaction is at present unknown. The mouse intestinal tract seems to excrete preferentially inorganic mercury as described previously for the rat. The relative amount of inorganic mercury is higher in the faeces than in any other organ related to the gastrointestinal tract. The total biliary excretion of mercury in the mouse is not known, so that a balance sheet for intestinal transport and excretion of mercury cannot be given. The concentration of mercury in the bile is high, but the major complexing agent is different in mice and rats (NORSETH & CLARKSON 1970b and c). The significance of this in the intestinal transport of mercury remains to be determined.

The blood and the kidneys contain a higher amount of the injected dose in the rat than in the mouse (BERGLUND & BERLIN 1969). Differences in biotransformation may explain differences in the kidney. The mouse kidney contained a relatively lower amount of inorganic mercury than the rat kidney. The difference is particularly evident some time after the injection. By assuming added to the mouse kidney the amount of inorganic mercury necessary to make the relative amount of inorganic mercury in rats and mice equal at the 22 days interval, the liver to kidney ratio would be the same in the two species. This may be related to differences in enterohepatic circulation of mercury since complexing to thiols decreases the stability of some organomercurials (WEINER *et al.* 1962; NORSETH & CLARKSON 1970b).

Higher excretion rates of mercury in mice than in rats indicate lower reabsorption rates in the intestinal tract. Different turn-over time of intestinal epithelium may be of importance. Further testing of the distribution and the stability of methyl mercury after injection of methyl mercuric cysteine into rats and mice, and the testing of enterohepatic circulation of mercury complexed in bile in the mouse are necessary to answer this question.

The present results support the assumption that inorganic mercury is not

the toxic agent at the site of action for the brain lesions which occur in methyl mercury intoxications, as discussed in a previous paper (NORSETH & CLARKSON 1970b).

Differences in *biotransformation* and bile excretion provide a possible explanation for the different organ distribution and excretion rates of methyl mercuric salts *in rats and mice. Toxicity hazards to man cannot be fully* understood without some knowledge of the pharmacokinetics of the toxic agent. The *present results underline the importance of testing more than one* species in such work.

Acknowledgements

I would like to thank Miss Ellen Heiberg for technical help in this work.

REFERENCES

Berglund, F. & M. Berlin: Risk of methyl mercury cumulation in man and mammals and the relation between body burden of methyl mercury and toxic effects. In: *Chemical fall-out: Current research on persistent pesticides* p. 258. (Miller, M. W. & G. G. Bergs eds.) Charles C. Thomas Publisher, Springfield, Illinois, 1969.

Berlin, M. & S. Ullberg: Accumulation and retention of mercury in the mouse. I. An autoradiographic study after a single intravenous injection of mercuric chloride. *Arch. environ. Hlth.* 1963a, **6**, 589–601.

Berlin, M. & S. Ullberg: Accumulation and retention of mercury in the mouse. III. An autoradiographic comparison of methyl mercuric dicyandiamide with inorganic mercury. *Arch. environ. Hlth.* 1963b, **6**, 610–616.

Clarkson, T. W., A. Rothstein & R. Sutherland: The mechanism of action of mercurial diuretics in rats; the metabolism of ^{203}Hg-labelled chlormerodrin. *Brit. J. Pharmacol.* 1965, **24**, 1–13.

Cross, I. M. & I. I. Pinean: Observations on the exchange between phenyl mercuric chloride and mercuric chloride using mercury-203. *J. amer. pharm. ass.* 1951, **40**, 95–97.

Gage, J. C.: Distribution and excretion of methyl and phenyl mercury salts. *Brit. J. ind. Med.* 1964, **21**, 197–202.

Miller, V. L., P. A. Klavano, A. C. Jerstad & Elisabeth Csonka: Absorption, distribution and excretion of phenyl mercuric acetate. *Toxicol Appl. Pharmacol.* 1960, **2**, 344–352.

Norseth, T. & T. W. Clarkson: Biotransformation of methyl mercury salts in the rat studies by specific determination of inorganic mercury. *Biochem. Pharmacol.* 1970a, **19**, 2775–2783.

Norseth, T. & T. W. Clarkson: Studies on the biotransformation of ^{203}Hg-labelled methyl mercury chloride in rats. *Arch environ. Hlth* 1970b, **19**, 2775–2783.

Norseth, T. & T. W. Clarkson: Intestinal transport of ^{203}Hg-labelled methyl mercuric chloride. Role of biotransformation in rats *Arch. environ. Hlth.* 1970c, in press.

Weiner, J. M., R. I. Levy & G. H. Mudge: Studies on mercurial diuresis. *J. Pharmacol. Exp. Therap.* 1962, **138**, 96–112.

Östlund, K.: Studies on the metabolism of methyl mercury and dimethyl mercury in mice. *Acta pharmacol. et toxicol.* 1969, **27**, suppl. 1.

Acta pharmacol. et toxicol. 1971, 29, 385–401.

From the Department of Pharmacology and Toxicology and the Department of Analytical Chemistry, Research Department of the KABI Group, Stockholm, Sweden

Metabolic Disposition of Emepronium in the Dog with a Note on the Relationship between Plasma Concentration and Effect

By

A. Sundwall, K. Uthne and J. Vessman

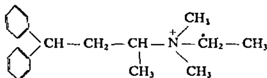
(Received October 14, 1970)

Abstract: The fate of emepronium (cetiprin®) in the dog has been studied following different routes of administration. The drug is rather extensively metabolized following both intravenous, oral and rectal administration. At least two metabolites are formed. Following intravenous injection twice as much radioactivity is found in the faeces as in the urine, about half in the form of the unchanged drug. This means that there is probably an extensive enterohepatic circulation of the drug. In four dogs 0.6, 0.8, 1.1 and 1.6 % of the dose was excreted unchanged in the urine within 24 hours following oral administration of 20 mg/kg. It is concluded from experiments with the radioactive compound that from 2 to 10 % of the dose is absorbed if metabolism and biliary excretion are taken into account. Plasma concentrations of emepronium in the nanogram range can be determined by gas chromatography of the oxidation product, benzophenone. A plasma concentration of about 20 µg/ml is lethal to both rats and dogs and the threshold concentration for an effect on the heart rate in the dog is about 100 ng/ml.

Key-words: Emepronium – quaternary anticholinergic – absorption – plasma concentrations – enterohepatic circulation

---

Emepronium bromide (cetiprin®) is an anticholinergic quaternary ammonium compound, which is used in the treatment of nocturnal frequency.



Although the clinical effectiveness of the drug is documented (BROCKLEHURST *et al.* 1969) it was considered to be important to investigate further the fate of the drug in the body by chemical methods and also to study the relationship between plasma concentration and effect. This approach was

made possible by the development of a sensitive gas chromatographic method for analysis of emepronium in plasma and urine (Vessman *et al.* 1970).

As judged from the amount of unchanged drug excreted in the urine in man about 1 % of the amount given orally seems to be absorbed. However, this could be an underestimate if the drug undergoes substantial metabolism and/or biliary excretion. Since it is known from studies in rats (Hansson & Schmiterlöw 1961) that radioactivity is excreted in the faeces to a great extent following intramuscular injection of the ^{14}C-labelled drug, particular attention was paid to establish the relative importance of biliary excretion.

A major part of the study has been performed with the ^{14}C-labelled compound.* The label is denoted by an asterisk in the formula shown above.

Methods

Animal experiments.

The majority of the experiments were performed in female dogs (beagles weighing 8–10 kg) who were trained for renal clearance experiments, e. g. to stand in a frame with catheters placed in the urinary bladder and in a superficial vein. In these experiments the electrocardiogram (ECG) was recorded intermittently in order to relate the heart rate to plasma concentrations.

Blood samples (10 ml) were collected in heparinized test tubes before the administration of emepronium and also after ¼, ½, 2, 4, 6 and 24 hrs respectively. The ECG was recorded for a few min. immediately before taking the blood samples.

The urine was collected at 2 hrs intervals during the first 6 hrs after which the dogs were placed in metabolism cages. The urine and faeces were then collected at 24 hrs intervals for 7 to 8 days (when insignificant amounts of emepronium were present in the urine). The cages were rinsed with 300 ml of water after the urine and faeces had been collected. The urine and faeces as well as the rinse water were deep frozen until analyzed.

Emepronium was given by intravenous, oral and rectal administration. An isotonic saline solution was used for intravenous injection, gelatine capsules for oral administration and suppositories (Witepsol H 15 as base) for rectal administration.

All three routes of administration were studied in each of three dogs at approximately 14 days intervals and ^{14}C-labelled emepronium was always used in the third experiment. The doses were 5 mg/kg (2 µci/kg) intravenously 20 mg/kg orally and 6.5 and 12 mg/kg (2.6 and 5.2 µci/kg) rectally.

In the rat experiments, emepronium was given orally (1100 mg/kg) as in aqueous solution (2 ml/kg). The animals were killed at different intervals and about 2 ml of blood were taken from the abdominal aorta.

Assay of radioactivity in plasma, urine and faeces.

Radioactivity in plasma, urine and rinse water was measured by means of liquid scintillation (Packard 3375) in a scintillation solution containing 960 ml dioxane, 5.12 g PPO, 0.128 g POPOP and 102.4 g naphtalene.

---

* Our thanks are due to Mr. S. Carlsson, head of the department of organic chemistry at Recip for synthesis of the labelled compound.

The faeces were homogenized by stirring and gradually adding 4 volumes of water. After 30 min. stirring the homogeneous suspension was centrifuged. The pellet was resuspended in 5 volumes of water and the suspension centrifuged. 0.2 ml of the combined supernatants were dried on filter paper and aliquots of the pellets freeze dried and carefully ground in a mortar. The paper strips and 30–40 mg aliquots of the freeze dried pellets were subjected to combustion in oxygen flasks and the $^{14}CO_2$ produced trapped in a scintillation solution containing 270 ml phenethylamine, 270 ml methanol, 460 ml toluene, 5 g PPO and 0.1 g POPOP (DAVIDSON & OLIVERIO 1967). It was found that only 50 % of the radioactivity in the faeces was present in the water extracts [the ratio between the radioactivity in the extract and pellet was 1.02 ± 0.17 (S. E. M.)]. The efficiency and reproducibility of the combustion procedure to be checked by the combustion of known amounts of radioactivity added to paper strips. The recovery was 91.1 % and the reproducibility ± 1.12 % (standard error of the mean of 10 experiments). Quench corrections were made by the channels ratio procedure.

Analysis of emepronium in plasma, urine and faeces extracts.

Analyses of emepronium in plasma, urine and faeces extracts were made with the benzophenone method (VESSMAN *et al.* 1970). The method comprises the following steps: Extraction of emepronium as ion pair with perchlorate into methylene chloride, oxidation of the emepronium perchlorate with chromic acid to benzophenone, and determination of the benzophenone formed with electron capture gas chromatography. Compounds with the gem-diphenyl-moiety intact are determined by this procedure while e.g. metabolites with an aromatic hydroxyl group are excluded (VESSMAN *et al.* 1970).

All samples were, if necessary, diluted to a concentration of about 50 ng/ml and 4.00 ml of this dilution taken for analysis.

The sensitivity limit for this method is about 5 ng/ml. Urine samples, when sample size allowed, were analyzed by a photometric procedure consisting of perchlorate ion pair extraction and determination of the emepronium content with bromothymol blue (EKSBORG *et al.* 1970). This method has a sensitivity of 1 µg/ml with a sample size of 15 ml. When urine samples were analyzed by both gas chromatography and photometry the values agreed within 5 %.

In order to check the proportion of unchanged emepronium in the faeces extracts and residues, the latter were dissolved in 6 N hydrochloric acid and analyzed with the gas chromatographic procedure. In the three faeces samples checked (dog 14 intravenously day 1, 2 and 3) the ratios between the emepronium content of water extract and residue were found to be 0.93, 0.90 and 0.99 respectively. Thus half of the emepronium in the faeces was recovered in water extracts.

This means that 50 % of both the radioactivity and the emepronium content of the faeces are present in the water extracts.

Separation of radioactive metabolites in urine by paper chromatography and high voltage electrophoresis.

Descending paper chromatography was performed with the upper phase of a mixture of n-butanol, ethanol, water (4 . 1 . 5) using Whatman No. 1 paper. High voltage paper electrophoresis was carried out in pyridine, acetic acid, acetone, water (40 : 40 : 300 : 1620) with a pH of 4.5 at 10° and a voltage drop of 50 volts/cm.

Unlabelled emepronium was frequently added to aliquots of urine before chromatography and electrophoresis in order to check the position of the unchanged drug. The spot was visualized with a modified Dragendorff reagent. The paper strips were

in a Packard radiochromatogram scanner. Control experiments revealed a counting efficiency of 23 %.

Separation of emepronium from a possible alcoholic metabolite.

Particular attention was paid to detect a possible hydroxylation of emepronium in the ethyl group to 2-hydroxyethyl (3,3-diphenyl-1-methyl-propyl) dimethylammonium bromide* since this compound might interfere in the gas chromatographic analysis of emepronium. It was found that the compound was indistinguishable from emepronium in the paper chromatographic and electrophoretic system used. However efficient separation was obtained by thin layer chromatography on cellulose impregnated with 0.015 N-$HClO_4$ as described by GRÖNINGSSON & SCHILL (1969). The mobile phase consisted of chloroform with 5 % 1-pentanol. The Rf-values were respectively 0.78 and 0.45 for emepronium and the alcohol. The spots were visualized with a Dragendorff's reagent. 0.25 µg of both compounds could be detected. In the urine samples studied the amount of emepronium were around 10 µg. This means that less than 2.5 % of the alcohol was present.

Results

Oral administration.

Lethal or nearly lethal plasma concentrations of emepronium were found to be in the range of 15–20 µg/ml following a single oral administration of the bromide to rats and dogs (table 1 and 2). Five out of 26 rats given 1100 mg/kg (water solution) died within 15 min. The three rats killed after 15 min. had plasma concentrations from 15.2 to 19.3 µg/ml. The rate of disappearance of emepronium from the plasma in the rat was initially very high (half-life

Table 1.

Plasma concentrations of emepronium in rats following oral administration of 1100 mg/kg (dissolved in water).

Time after administration hrs	Plasma concentration µg/ml
0.25	16.9 (19.3 15.2, 16.3)*
0.5	2.6 (2.1, 4.0, 1.8)
1	2.8 (2.5, 4.0, 2.0)
2	0.86 (0.56, 1.03, 0.99)
4	0.32 (0.36, 0.35, 0.26)
6	1.05 (0.96, 1.2, 0.97)
24	0.30 (0.22, 0.42, 0.26)

* Out of the 26 rats given emepronium 5 died within 15 min.

* Our thanks are due to Mr. Nils Forsberg for the synthesis of the compound.

Table 2.

Plasma concentrations of emepronium in dogs following oral administration of toxic dose (gelatine capsules).

Time hrs	Plasma concentration (μg/ml)				
	175 mg/kg		250 mg/kg		500 mg/kg
½	2.7	7.5	15.5	38.0	12.0
1	3 2	8 6	Dead	Dead	17.3
2	3.2	5.5			Dead
4	3.4	3.9			
6	3 3	3.3			
24	-	2.4			

5–10 min.). The half-life was then about 60 min. from 1 to 4 hrs. Between 4 and 6 hrs the plasma concentration increased from 0.3 to 1.1 μg/ml. After 24 hrs the plasma concentration was about 0.3 μg/ml.

In the dog 250 mg/kg proved to be lethal when given orally in the form of gelatine capsules. Death ensued within 1 hr and the plasma concentrations shortly before death were 15.5 and 38 μg/ml. With 500 mg/kg the plasma

Table 3.

Plasma concentrations and urinary excretion of unchanged emepronium in dogs after oral administration of 20 mg/kg of the bromide (gelatine capsules).

Time hrs	Plasma concentration ng/ml						
	Dog 21	Dog 14	Dog 22	Dog 25	Dog 28	Dog 29	Dog 30
¼	-	68	<5.0	<5.0	8	1494	72
½	59.6	50	15.7	11	8	643	109
1	146	38	42.6	-	31	154	60
2	87.4	34	269	24.6	28	93	238
4	37.6	27	94.7	41.7	8	125	60
6	43.2	20	59 0	11.9	1	66	23
24	10.2	6.5	<5.0	8 6	-	-	-
Per cent excreted unchanged in urine in 24 hrs	1.0	0.6	1.6	0.8	-	-	-

Table 4.

Excretion of radioactivity in urine and faeces following intravenous, oral and rectal administration of ^{14}C-labelled emepronium bromide. (5 mg/kg intravenously, 20 mg/kg orally, 6.5 mg/kg rectally). Figures in brackets represent the amounts of faeces in grams.

Time days	% of administered dose								
	Dog 14 Intravenous			Dog 25 Oral			Dog 22 Rectal		
	Urine	Rinse	Faeces	Urine	Rinse	Faeces	Urine	Rinse	Faeces
0–1	26.6	0.6	24.0 (44)	1.9	0.8	54.6 (194)	2.8	0.3	20.4 (63)
1–2	0.4	0.1	12.0 (47)	0.1		0.6 (23)	0.3	0.1	26.1 (115)
2–3	0.7		10.1 (34)	0.1		0.1 (13)	0.1	0.1	3.4 (60)
3–4	0.4		}	0.1		}	0.1		}
4–5	0.2		13.7 (348)	0.1		0.7 (274)	0.1		1.0 (379)
5–7	0.2		}	0.1		}	0.1		}
Total	28.5	0.7	59.8 (473)	2.4	0.8	56.0 (504)	3.5	0.5	50.9 (617)

Table 5.

Urinary excretion of ^{14}C and unchanged drug following different routes of administration of ^{14}C-labelled emepronium bromide.

	Hrs	^{14}C % of administered dose	Unchanged drug % of administered dose	% of radioactivity as metabolites
Dog 14 Intravenously (5 mg/kg)	0– 2 2– 4 4– 6 6–24	11.8 6.0 2.6 6.2	7.1 0.8 0.5 1.0	40 87 81 83
	0–24	26.6	9.4	65
Dog 25 Orally (20 mg/kg)	0– 2 2– 4 4– 6 6–24	0.1 0.4 0.6 0.8	0.05 0.17 0.19 0.29	50 57 69 64
	0–24	1.9	0.70	63
Dog 22 Rectally (6.5 mg/kg)	0– 2 2– 4 4– 6 6–24	0.0 1.5 0.3 1.0	0.0 0.6 0.1 0.1	- 60 66 90
	0–24	2.8	0.8	71
Dog 25 Rectally (12 mg/kg)	0– 2 2– 4 4– 6	0.2 0.2 0.1	0.04 0.01 0.01	80 95 90

concentration was 17.3 μg/ml shortly before death. 175 mg/kg of the drug produced almost complete anuria during the first 24 hrs. Maximum plasma concentrations were reached after 1 hr (3.2 and 8.6 μg/ml). Emepronium was then slowly cleared from the plasma. In one of the dogs the plasma concentration was practically constant i. e. from 1 to 6 hrs after administration.

Table 3 shows the results in 7 dogs after the oral administration of 20 mg/kg in gelatine capsules. As seen in the table, there are rather marked individual differences in plasma concentration, peak values ranging from 42 to 1494 ng/ml. From 0.6 to 1.6 % of the dose was recovered unchanged in the urine after 24 hrs.

^{14}C-labelled emepronium was used in one of the experiments (dog 25, table 4 and 5). It can be seen that 1.9 % of the administered radioactivity was excreted in the urine in 24 hrs. Since the amount of drug excreted unchanged was 0.7 % of the administered emepronium, 58 % of the excreted radioactivity was in the form of metabolites. In the samples collected from 2 to 6 hrs after administration of the drug the metabolites represented 57 to 69 % of the radioactivity.

From determinations of radioactivity and emepronium (gas chromatography) in plasma it was calculated that 34 % of the radioactivity in the plasma was in the form of emepronium 4 hrs after administration of the drug. The corresponding figure was 11 % after 6 hrs.

Table 4 shows that almost all the radioactivity excreted in the faeces (56 % of administered) appeared in the first 24 hrs sample. In the faeces extract, 94 % of the radioactivity was in the form of the unchanged drug (table 6).

Intravenous injection.

The plasma concentrations obtained following intravenous administration *of 5 mg/kg* are shown in fig. 1. It can be seen that the drug is rather rapidly

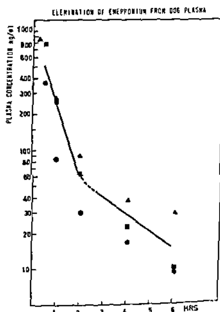


Fig. 1. Plasma concentrations of emepronium in three dogs following intravenous injection of 5 mg/kg. The dogs are denoted by ● ■ ▲.

Table 6.

Amount of ^{14}C and unchanged drug in faeces extract following different routes of administration of ^{14}C-labelled emepronium bromide.

	Days	^{14}C % of administered dose	Unchanged drug % of administered dose	% of radioactivity as metabolites
Dog 14	0–1	11.0	3.0	73
Intravenously	1–2	6.1	4.1	33
(5 mg/kg)	2–3	3.7	2.0	46
Dog 22	0–1	9.6	11.2	0
Rectally	1–2	8.7	7.8	10
(6.5 mg/kg)	2–3	2.0	1.7	15
Dog 25	0–1	19.5	18.2	6
Orally	1–2	0.5	0.2	40
(20 mg/kg)				

cleared from the plasma during the first two hrs (half-life 15 min.) after which the rate of disappearance is considerably slower (half-life about 2 hrs).

From 5.0 to 9.4 % of the injected drug was found unchanged in the urine 24 hrs after injection. The major part (80–84 %) was excreted within 4 hrs.

One dog (No. 14) was injected with the ^{14}C-labelled drug. 27 % of the injected radioactivity was recovered in the urine in 24 hrs. The corresponding figure for unchanged drug was 9.4 %. This means that 65 % of the radioactivity excreted in the urine in 24 hrs was in the form of metabolites.

About 60 % of the injected radioactivity was excreted with the faeces in 7 days (table 4). About half of the radioactivity in the faeces extract represented unchanged emepronium (table 6).

The relationship between radioactivity and unchanged drug in the plasma is shown in fig. 2. It can be seen that about 46 % of the radioactivity in the plasma is in the form of emepronium 30 min. after the injection. Between 3 and 6 hrs the corresponding figure is about 10 %.

Rectal administration.

Plasma concentrations following rectal administration (suppositories) are shown in table 7. From 0.8 to 2.4 % of the administered dose was recovered in the urine after 24 hrs.

^{14}C-labelled emepronium was used in two of the experiments (dog 22 and 25). 2.2 and 2.8 % of the administered radioactivity were recovered in th

urine in 24 hrs. A comparison with the excretion of the unchanged drug shows that from 60–95 % of the radioactivity is in the form of metabolites (table 5).

51 and 72 % of administered radioactivity were found in the faeces. In one of the dogs (table 4) the major part was recovered during the first two days while in the other dog, 85 % of the amount found in the faeces was recovered between the 3rd and 7th day. Practically all the radioactivity in the faeces was due to unchanged emepronium (table 6). In dog 22 2.8 % of the administered radioactivity was recovered in the urine in the first 24 hrs. Although only 0.3 % was found in the urine during the next 24 hrs, the amount in the faeces was about the same (20 and 26 %) during the two consecutive 24 hrs periods (table 4).

Separation of urinary metabolites.

From the comparisons between radioactivity and unchanged drug in the plasma and urine it was evident that emepronium is rather rapidly metabolized. The amount of radioactivity excreted in the urine in the form of

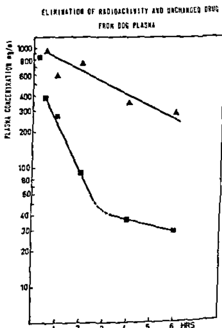


Fig. 2. Plasma concentration of emepronium after intravenous injection of the ^{14}C-labelled drug (Dog 25, 5 mg/kg) as calculated from radioactivity and by analysis by gas chromatography. ▲ Calculated from radioactivity. ■ Gas chromatography.

Table 7.

Plasma concentrations and urinary excretion of emepronium in dogs after rectal administration of 6.5 and 12 mg/kg of the bromide (suppositories).

Time hrs	Plasma concentration (ng/ml) Dog 22 6.5 mg/kg	Dog 14 12.5 mg/kg	Dog 25 12.5 mg/kg
¼	40	136	37
½	43	19.6	6.6
1	5	15.2	12
2	< 5	7.1	< 5
4	< 5	10.0	14
6	< 5	6.6	< 5
24	< 5	1.7	–
Per cent excreted unchanged in urine in 24 hrs	0.8	1.1	< 2.4*

* Contaminated with faeces.

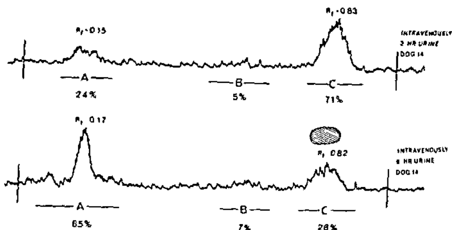
metabolites was about the same (60–70 %) following all three routes of administration (table 5).

The results of separation by paper chromatography and high voltage paper electrophoresis of radioactive urinary metabolites in some intravenous experiments are shown in fig. 3. It can be seen that three peaks appear, one of which is unchanged emepronium. The relative proportions were determined by liquid scintillation after combustion of paper strips from the chromatograms and electropherograms and by planimetry after scanning in a chromatogram scanner. Similar patterns were observed when the urine was collected after other routes of administration were studied.

In the gas chromatographic procedure compounds hydroxylated in the aliphatic part of the molecule could be determined as emepronium. A possible metabolite of this kind (2-hydroxyethyl) (3.3-diphenyl-1-methyl-propyl) dimethyl ammonium bromide was synthetized. Separation of emepronium from the alcohol could be achieved on thin layers of cellulose impregnated with perchloric acid. The alcohol could not be distinguished from emepronium in the paper chromatographic and electrophoretic systems used. When urine samples were extracted as described in the gas chromatographic procedure and examined in the thin-layer chromatographic system, there was evidence for the presence of this alcoholic compound. Control

SEPARATION OF METABOLITES IN URINE (DOG)

PAPER CHROMATHOGRAPHY



PAPER ELECTROPHORESIS

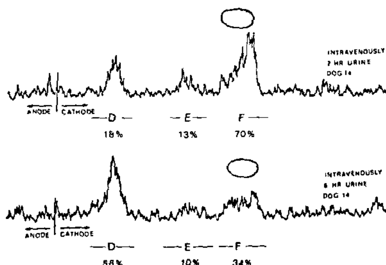


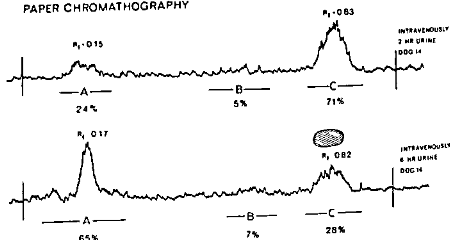
Fig. 3. Separation of radioactive metabolites in urine by high voltage paper electrophoresis and paper chromatography. Evaluation made in a Packard radiochromatogram scanner. The part of paper analyzed by combustion is indicated in the figure (A–F) The spot indicates added unlabelled emepronium.

Table 8.

Relationship between plasma concentration and heart rate following administration of emepronium by different routes (Dog). H. r. = Heart rate.

Time	Dog 25 Rectal		Dog 14 Rectal		Dog 22 Oral		Dog 30 Oral		Dog 29 Oral		Dog 28 Oral	
	Plasma conc. ng/ml	H. r. Beats/ min.	Plasma conc. ng/ml	H r. Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.
0	–	108	–	126	–	72	–	112	–	82	–	108
15 min.	37.2	108	136	162	< 5	96	72	96	1494	216	8	90
30 min.	6.6	108	19.6	132	15.7	78	109	135	643	204	8	108
1 hr	11.9	78	15.2	126	42.6	108	60	120	154	114	31	102
2 hrs	3.2	96	7 1		269	150	238	162	93	66	28	138
4 hrs	14 6	74	10.0	108	94.7	108	60	90	125	84	8	108
6 hrs	< 5	74	6.6	114	59 0	78	23	60	66	84	< 5	84
24 hrs	–		1.7		< 5							

SEPARATION OF METABOLITES IN URINE (DOG)



PAPER ELECTROPHORESIS

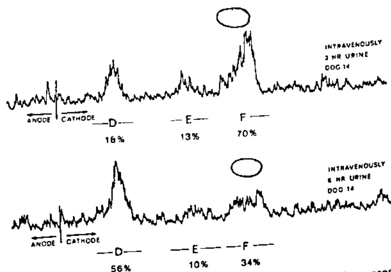


Fig. 3. Separation of radioactive metabolites in urine by high voltage paper electrophoresis and paper chromatography. Evaluation made in a Packard radiochromatogram scanner. The part of paper analyzed by combustion is indicated in the figure (A-F). The spot indicates added unlabelled emepronium.

Table 8.

Relationship between plasma concentration and heart rate following administration of emepronium by different routes (Dog).
H. r. = Heart rate.

Time	Dog 25 Rectal		Dog 14 Rectal		Dog 22 Oral		Dog 30 Oral		Dog 29 Oral		Dog 28 Oral	
	Plasma conc. ng/ml	H. r Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.	Plasma conc. ng/ml	H. r Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.	Plasma conc. ng/ml	H. r. Beats/ min.
0	–	108	–	126	–	72	–	112	–	82	–	108
15 min.	37.2	108	136	162	< 5	96	72	96	1494	216	8	90
30 min.	6.6	108	19.6	132	15.7	78	109	135	643	204	8	108
1 hr	11.9	78	15.2	126	42.6	108	60	120	154	114	31	102
2 hrs	3.2	96	7.1		269	150	238	162	93	66	28	138
4 hrs	14.6	74	10.0	108	94.7	108	60	90	125	84	8	108
6 hrs	< 5	74	6.6	114	59.0	78	23	60	66	84	< 5	84
24 hrs	–		1.7		< 5							

showed that the alcohol would have been detected in amounts corresponding to less than 2.5 % of the emepronium present. Separation was also performed after acid hydrolysis in order to split any possible conjugates.

Relationship between heart rate and plasma concentration.

The heart rate was measured in nine experiments by ECG recording. The results are summarized in table 8, which also shows the plasma concentrations in samples taken immediately afterwards. It is evident from the table that the threshold concentration in the plasma which causes an increase in heart rate is about 150–250 ng/ml.

Discussion

It is well known that intestinal absorption of quaternary ammonium compounds is rather slow and far from complete. In the case of benzomethamine and banthine from 0.8 to 8.3 % and 0.2 to 3.4 % of an oral dose were found unchanged in the urine in 4 hrs (dog) (Levine & Clark 1955). In the present study with emepronium the corresponding figures were 0.6 to 1.6 %. However, the excretion of unchanged drug in the urine is not a very reliable parameter for the study of intestinal absorption, if the drug undergoes significant metabolism and/or biliary excretion. With regard to the metabolism of emepronium there appears to be interesting species differences. In the rat it is metabolized only to a minor extent (Hansson & Schmiterlöw 1961), while it undergoes much more extensive metabolic transformation in the dog (about 60–70 % of the radioactivity in the urine and faeces are in the form of metabolites following intravenous administration).

With both methods of separation of urinary metabolites (chromatography and electrophoresis) two metabolites were found. The proportions of unchanged drug in the urine obtained with the two methods agree rather well (71 to 70 and 28–34 %). A comparison with the values found by gas chromatography reveal somewhat lower figures (60 and 19 % respectively). This difference may be explained by the fact that the percentage unchanged drug in the case of paper chromatography and electrophoresis is calculated from the sum of the radioactivity found in the three peaks, while in the case of gas chromatography it is calculated from the total radioactivity in the sample.

A comparison of the percentage of radioactivity present as emepronium in plasma and urine shows that there appears to be no preferential urinary elimination of the metabolites (the proportion is almost the same in plasma and urine).

Extensive biliary excretion is also indicated by the present investigation.

Following intravenous injection 60 % of the label was excreted in the faeces during the 7 days of observation. It was found that from 27 to 66 % was in the form of the unchanged drug. The rat experiments indicate an enterohepatic circulation of the unchanged drug. There was a marked increase in plasma concentration between 4 and 6 hrs following oral administration. A similar tendency was also seen in the dogs (table 3 and 7). From our experiments it cannot be stated that the drug is excreted in the bile. It might as well be secreted through the gastrointestinal mucosa. However, the latter possibility seems less likely to be of importance since the experiments of HANSSON & SCHMITERLÖW (1961) demonstrated extensive biliary excretion of radioactivity in rats with bile duct cannula. It is also known from experiments with other monoquaternary ammonium compound that the biliary excretion of the unchanged drug is a major route of elimination (LEVINE & CLARK 1955 and 1957). In man 14 % of an intramuscular dose of benzomethamine is excreted unchanged in the bile (bile duct fistula) within 4 hrs (LEVINE & CLARK 1955).

Thus, when metabolism and biliary excretion are accounted for, the gastrointestinal absorption of emepronium in the dog is much more efficient than is indicated by the urinary excretion of the unchanged drug. If 1.1 % of the administered dose is found unchanged in the urine, the total amount absorbed would be about 6 %. This conclusion has been reached by the following reasoning: Following intravenous injection, about twice as much label is excreted in the faeces as in the urine. Thus, one would expect a similar fraction of a dose absorbed from the intestine to be excreted in the faeces. Since 2.5 % of the administered radioactivity was excreted in the urine, another 4 % would be excreted in the faeces. Thus it is suggested that the amount absorbed is approximately 6 times the amount excreted unchanged in the urine. One would possibly expect an even larger proportion to be excreted in the bile in the case of oral administration, as in this case all the absorbed material passes through the liver.

In the dog, rectal absorption from suppositories seems to be of the same order as following oral administration of gelatine capsules, as judged by the amount of label excreted in the urine. However, in man rectal absorption appears to be much more efficient than oral administration since similar plasma concentrations are produced by doses differing by a factor of three (VESSMAN *et al.* 1970). The reason for this discrepancy is unknown but may be related to the fact that the hemorrhoidal venous plexus is rather rudimentory in the dog (MILLER *et al.* 1964).

The specificity of the gas chromatographic method used for analysis of emepronium in the plasma was investigated and the results are described in a previous paper (VESSMAN *et al.* 1970). It was shown that phenols and dealkylation products do not interfere in the analysis. On the other hand, it

was believed that alcohol metabolites might be determined as emepronium if present. In the present paper the presence in the urine of one of these possible alcoholic metabolites (emepronium hydroxylated in the ethyl group) was investigated, but could not be detected in the urine from dogs treated with emepronium.

With the benzophenone method, it has been possible to analyse very low concentrations of emepronium in the plasma (5 ng/ml). It has therefore been possible to study the relationship between the plasma concentration and pharmacological effects.

Due to the short half-life of the drug in plasma ($t_{1/2}$ about 2 hrs) it was difficult to obtain steady state conditions. Instead, single oral and rectal administration were used. The plasma concentrations and heart rate were measured simultaneously at rather short intervals. It was found that an increased heart rate occurred when the plasma concentration was about 150–250 ng/ml.

Studies are now in progress to study further the pharmacokinetics of emepronium in man in order to relate plasma levels to pharmacological effects.

It is interesting to note that following a lethal dose to rats and dogs the plasma concentrations were almost the same (17 and 24 μg/ml) although the LD50 differed markedly between the rat and dog (1100 and 250 mg/kg).

Acknowledgement

We would like to thank Mr. Stig Norrman, Mr. Bengt Osanius and Mrs. Signhild Strömberg for skilled technical assistance.

REFERENCES

Brocklehurst, J. H., J. B. Dillane, J. Fry & P. Armitage: Clinical trial of emepronium bromide in nocturnal frequency of old age. *Brit. med. J.* 1969, 2, 216.

Davidson, J. D. & V. T. Oliverio: Tritium and carbon-14 by oxygen flask combustion. *Atomlight* 1967, No 60.

Eksborg, S., B.-A. Persson, J. Vessman & B. Enell: Determination of a quaternary ammonium compound, emepronium bromide (Cetiprin®) in human urine by an ion pair extraction method. *J. Pharm. Sci.* 1971, in press.

Gröningsson, K. & G. Schill: Thin layer chromatography of ion pairs on impregnated layers. *Acta Pharm. Suecica* 1969, 6, 447.

Hansson, E. & C. G. Schmiterlöw: Biological fate of ^{14}C-labelled ethyl (3,3-diphenyl-1-methyl-propyl) dimethylammoniumbromide, Cetiprin®, a monoquarternary ammonium compound. *Acta pharmacol. et toxicol.* 1961, 18, 183.

Levine, R. M. & B. D. Clark: The biotransformation, excretion, and distribution of the anticholinergic quaternary ammonium compound benzomethamine, N-diethylaminoethyl-N'-methyl-benzilamide methobromide (MC 3199) and its tertiary amine analogue (MC 3137) and related compounds in animals. *J. Pharmacol.* 1955, 114, 63–77.

Levine, R. M. & B. D. Clark: The physiological disposition of oxophenomium bromide (antrenyl) and related compounds. *J. Pharmacol.* 1957, 121, 63–70.

Miller, M. E., G. C. Christensen & H. E. Evans: Anatomy of the dog. *W. B. Saunders Company*, Philadelphia, 1964.

Vessman, J., S. Strömberg & G. Rietz: Gas chromatography and electron capture detection of benzophenone formed by chromic acid oxidation. II Determination of emepronium (Cetiprin®) a quaternary ammonium compound in serum. *Acta Pharm. Suecica* 1970, 7, 363–372.

Acta pharmacol. et toxicol. 1971, 29, 402–416.

From the Departments of Pharmacology and Pathology, University of Oulu, Finland

Effect of Carbon Tetrachloride Induced Progressive Liver Damage on the Metabolism of Hexobarbital and Bilirubin *in Vivo*

By

Martti Vorne and Martti Alavaikko

(Received October 28, 1970)

Abstract: Rats were given 2 ml carbon tetrachloride per kg body weight subcutaneously 3 times a week. The development of liver changes was observed from histological specimens, and changes in the functional capacity of the liver were determined by measuring the hexobarbital sleeping time and the retention of bilirubin given to animals after carbon tetrachloride administration for 2, 4, 8, and 16 weeks. The animals receiving carbon tetrachloride gained weight more slowly, and their growth ceased shortly after two months of treatment. The relative liver weights of the rats receiving carbon tetrachloride did not differ from those of the controls after administration for 2 weeks, but after 4 weeks the relative liver weights of those rats receiving carbon tetrachloride were 12%, after 8 weeks 29%, and after 16 weeks 65% greater. It was noted that carbon tetrachloride had induced progressive morphological liver changes. Cirrhosis was apparent after 8 weeks of treatment and after 16 weeks it was severe. After 8 weeks of carbon tetrachloride administration the retention of exogenous bilirubin also increased, being 76 % greater than in the controls. After 16 weeks of administration, bilirubin retention was 94% greater than in the controls. The hexobarbital sleeping time increased progressively: after 2 weeks it was twice as long as in the controls, after 4 weeks 2½ times, after 8 weeks more than 3 times, and after 16 weeks approximately 10 times as long as in the controls.

Key-words: Carbon tetrachloride – liver damage – metabolism *in vivo* – hexobarbital – bilirubin.

Most foreign substances are metabolized by microsomal liver enzymes. It has been assumed that liver diseases impair drug metabolism (BUSH 1963; SELIGSON 1963). So far, only a few studies have been made which confirm this assumption. A few clinical investigations have been carried out, but their results are somewhat contradictory. The half-lives of phenylbutazone, salicylic acid, aminopyrine (aminophenazonum NFN), dicumarol and antipyrine (phenazonum NFN) in patients with Laennec's cirrhosis were not signi-

ficantly different from the corresponding values in the controls (Brodie *et al.* 1959). Another clinical study confirmed the finding that the half-life of phenylbutazone was not significantly different between the controls and the patients with liver disease. However, when these patients were grouped according to whether or not they were pretreated with drugs, significantly longer half-lives were noted in the patients not undergoing drug treatment (Levi *et al.* 1968). Animal experiments have shown that obstructive jaundice impairs drug metabolism both *in vitro* (McLuen & Fouts 1961; Metge *et al.* 1964; Kaltiala 1971) and *in vivo* (McLuen & Fouts 1961). Experimental liver cirrhosis has provided contradictory results concerning changes in glucuronyl transferase activity. In one study it was shown that glucuronyl transferase activity remained almost unchanged (Beck *et al.* 1967), while in another the activity was found to decrease clearly (Metge *et al.* 1964).

By treating laboratory animals chronically with carbon tetrachloride, it is possible to induce liver damage which becomes gradually more severe, and finally ends in cirrhosis (Cameron & Karunaratne 1936; Kaufmann 1953; Islami *et al.* 1958; Wong & Finchk 1965; Olsson 1966) which histopathologically resembles the Laennec type of cirrhosis found in man (Kaufmann 1953). The investigations carried out so far have been concerned mainly with morphological changes and our knowledge of the ability of the cirrhotic liver to metabolize foreign substances is inadequate.

In the present study we have attempted to ascertain whether there is a correlation between the morphological severity of liver damage and the activity of liver microsomal enzymes. We chose hexobarbital (enhexymalum NFN), which is hydroxylated (Cooper & Brodie 1955), and bilirubin, which is conjugated (White 1967) in microsomes, as test substances. The investigations were made *in vivo* by measuring the duration of action of hexobarbital and the retention of bilirubin in the animals.

Material and methods

This study belongs to a series of experiments in which we used a total of 660 male Sprague-Dawley rats which weighed 90–160 g at the beginning of the experiments. They all came from the same farm (The Mankkaa Research Laboratory, Orion Oy, Finland). The animals were given normal laboratory food (The Hankkija Fodder Mixture, Hankkija Central Cooperative, Finland) and water *ad libitum* throughout the experiment.

The rats were given 2 ml carbon tetrachloride (E. Merck A. G., Darmstadt) per kg body weight subcutaneously 3 times a week. The control animals received a correspondingly physiological saline solution. After 2, 4, 8 and 16 weeks of treatment the hexobarbital sleeping time, bilirubin retention and morphological liver changes were recorded. These examinations were carried out 2 days after the last

Morphological methods.

In order to obtain the liver specimens, we did not kill the animals, but merely subjected them to approximately 70 % liver resection by removing the left lateral and the median lobes under light ether anaesthesia (HIGGINS & ANDERSON 1931). The liver specimens thus obtained were weighed and fixed in 10 % formalin. They were cut into 6 μ histological sections, which were stained with haematoxylin-eosin, van Gieson's stain and Turnbull's blue. In addition, frozen sections were stained with oil Red O in order to visualize the fat. The sections were examined under a light microscope In the histological examination, attention was paid to signs of parenchymal damage (degeneration and necrosis of parenchymal cells), regenerative changes (mitoses, multinuclear cells, regenerative nodes), and the development of fibrosis. In each time group 4–6 livers were examined.

Criteria of cirrhosis.

The following criteria of cirrhosis were used (Fifth Panamerican Congress of Gastroenterology: Report of the Board for Classification and Nomenclature of Cirrhosis of Liver, 1956, cited from OLSSON 1966):

1) All parts of the liver are involved, without necessarily affecting each lobule.
2) Cellular necrosis is present at some stage of the disease.
3) Nodular parenchymal regeneration.
4. Diffuse fibrosis.
5. Disorganization of the lobular architecture with connective tissue bands uniting centro-lobular zones with the portal tracts.

Hexobarbital sleeping time.

The hexobarbital sleeping time was measured by giving the rats 75 mg of hexobarbital (enhexymalum NFN, hexobarbital sodium, Siegfried S. A., Switzerland) per kg body weight intraperitoneally, and by measuring the time between the injection and the return of the righting reflex.

Bilirubin overload test.

The bilirubin overload test was performed according to a slight modification of the method of KRUEGER & HIGGINSON (1961). Fifty mg unconjugated bilirubin (Fluka A. G., Buchs, Switzerland) was dissolved into 10 ml isotonic Na_2CO_3-NaCl solution (0.5 g Na_2CO_3, 0.52 g NaCl/100 ml). Bilirubin (20 mg per kg body weight) was injected into the tail vein and the serum bilirubin content of a blood sample drawn from the tail 30 minutes later, was measured by the micromethod of WHITE *et al.* (1958). The bilirubin basal values were also determined by using the blood samples drawn from the tail. Haemolyzed samples were not used.

Statistics.

Student's t-test was used in the statistical analysis of the results.

Results

Body weight and condition of animals.

The animals receiving carbon tetrachloride gained weight more slowly than the controls, and their increase in weight ceased after 2 months (*table 1*). The

Table I.

Effect of chronic CCl_4 administration on body weight and "relative liver weight". The number of rats is given in brackets. The weight of the resected median and left lobes of liver (about 70 %) was used in calculations of the "relative liver weight". Results are expressed as mean of the values ± S. D.

Duration of treatment	Body weight (g)	Weight gain % of initial weight	Relative weight of the resected liver lobes (g/100 g body weight)	% of control
Beginning				
control	131 ± 24 (21)	–	–	–
CCl_4	139 ± 11 (21)	–	–	–
2 weeks				
control	187 ± 20 (21)	43	3.00 ± 0.22 (20)	100
CCl_4	189 ± 15 (21)	35	3.11 ± 0.27 (20)	104
4 weeks				
control	247 ± 16 (21)	88	2.72 ± 0.19 (20)	100
CCl_4	220 ± 18 (21)[a]	58	3.05 ± 0.29 (20)[a]	112
8 weeks				
control	304 ± 19 (21)	132	2.56 ± 0.23 (8)	100
CCl_4	252 ± 24 (21)[a]	81	3.31 ± 0.40 (8)[a]	129
16 weeks				
control	344 ± 35 (21)	162	2.70 ± 0.16 (8)	100
CCl_4	255 ± 34 (21)[a]	83	4.46 ± 0.45 (8)[a]	165

a) = $P < 0.001$.

mean weight of the 108 rats alive after the 16-week administration of carbon tetrachloride was 266 g (range 162–345 g). The 16-week group included 48 control rats, the mean weight of which was 346 g (range 258–412 g). Some of the rats receiving carbon tetrachloride developed local skin necroses at the beginning of injection treatment. Mortality in the carbon tetrachloride group was low during the first three months. In the 2-, 4- and 8-week groups only 0.7% (2 rats) died from injection complication, but in the 16-week group the mortality was 28% (42 rats). Most of these animals died during the last 2 weeks, apparently from severe liver cirrhosis. The control rats continued to gain weight throughout the experiment, and none of them died.

Weight of the livers.

The relative weights of the resected liver lobes increased in the rats receiving carbon tetrachloride as compared with the controls. After 2 weeks the difference was not significant (4%), but after 4 weeks the ones receiving carbon

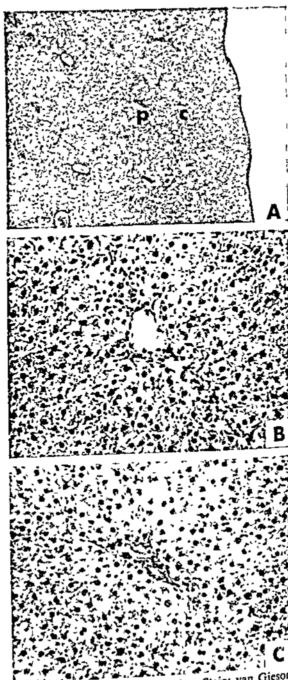


Fig. 1. Rat liver after 2 weeks CCl_4 administration. Stain: van Gieson. A. Architecture slightly disturbed. The surface of the liver is slightly rough. Magnification × 40. B. Centrolobular zone. Feathery degeneration and necroses of individual cells. Some mitoses. Magnification × 250. C. Portal and periportal tract. Parenchymal degeneration not so pronounced as in the centrolobular zone. Magnification × 250. c = central vein, p = portal tract.

tetrachloride had significantly (12%) greater relative weights, and the difference was further increased in the 8-weeks (29%) and 16-week (65%) groups (table 1).

Pathological changes of the livers.
Macroscopic findings.
The livers of the rats receiving carbon tetrachloride became lighter in colour and firmer. After 2 weeks of carbon tetrachloride administration, the livers were still macroscopically smooth-surfaced, but after that they gradually became rougher in texture. The livers of the rats which died towards the end of the treatment, and the livers of those treated for 16 weeks, were large, pale, hard and granular. At this stage most of the rats receiving carbon tetrachloride also had ascites.

Microscopic findings
1. *Carbon tetrachloride for 2 weeks (fig. 1).*
The general architecture of the liver had been preserved relatively well. The central veins and the portal tracts were clearly distinguishable. Considerable disorganization of parenchymal cells, feathery degeneration, and necroses of individual cells were noted, particularly in the centrolobular area and around the collecting veins. Drops of fat were seen in some cells. The sizes of cells and nuclei varied considerably. Mitoses and binuclear cells were often seen. The wall of the central vein was slightly fibrotic, and around it there appeared weak fibroblast proliferation and a few thin fibrous bands oriented towards the periphery. Parenchymal cells in the periportal areas* showed slight swelling, but no clear increase of connective tissue was noted in the portal tracts.

2. *Carbon tetrachloride for 4 weeks (fig. 2).*
The general architecture was moderately disturbed and the beginning of pseudolobulation was noted in some places. Thin fibrous bands often united the central veins with each other and with the collecting veins. The walls of the central veins were already marked fibrotic and thickened, and there was abundant fibroblastic proliferation. Even at this stage the portal tracts still seemed more or less intact. Slight fibrosis was noted in some portal tracts, but the fibrous bands uniting the central veins and portal tracts were not yet present. Necroses and mitotic figures were numerous in the whole area of liver parenchyma, and most numerous in the centrolobular areas. A little more fat was seen than in the 2-week group.

* Periportal area, as used here, refers to the liver parenchyma bordering on tracht.

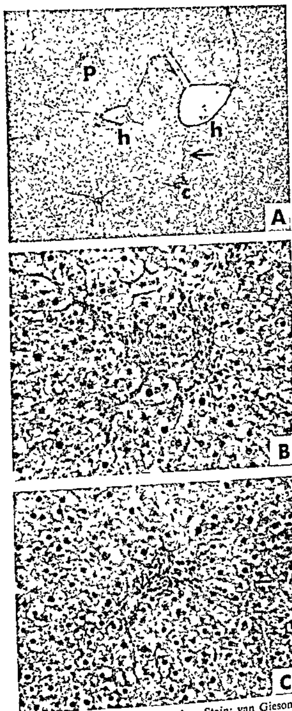


Fig. 2. Rat liver after 4 weeks CCl_4 administration. Stain: van Gieson. A. Architecture moderately disturbed. Magnification × 40. B. Marked fibroblastic proliferation in the wall and around the central vein. Magnification × 250. C. Considerable parenchymal degeneration in the periportal area. A very slight fibrosis in the portal tract. Magnification × 250. c = central vein, p = portal tract, h = a branch of the hepatic vein, arrow = a thin fibrous trabecula connecting a central vein to a branch of the hepatic vein.

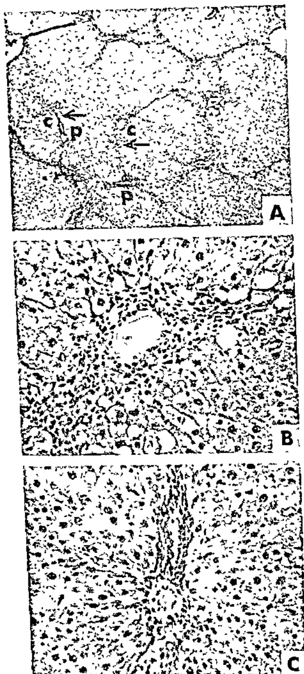


Fig. 3. Rat liver after 8 weeks CCl_4 administration. Stain: van Gieson. A. Architecture markedly disturbed. Histological picture satisfies the criteria of cirrhosis. Magnification × 40. B. Marked fibrosis in the centrolobular zone. Numerous fat droplets in the parenchyma. Magnification × 250. C. Portal and periportal tract. Fibrosis not so pronounced as in the centrolobular zone. Magnification × 250. c = central vein, p = portal tract, arrows = thin fibrous trabeculae connecting central portal tracts.

3. *Carbon tetrachloride for 8 weeks (fig. 3).*

The changes satisfied the above mentioned criteria of cirrhosis. Thick connective tissue septa rich in fibroblasts united the central veins with each other and with the collecting veins; this created pseudolobules which often had a portal tract in the middle. In some places it was already possible to discern fibrous bands uniting the portal tracts and fibrotic centrolobular areas. In addition to fibroblasts, granulocytes and a few pigmented macrophages were also seen in the fibrotic areas. The pigment was haemosiderin. Necroses of individual cells and mitoses were numerous. The sizes of cells and nuclei varied considerably, and a great number of binuclear cells were seen. No regular liver cell columns were found, the parenchymal cells being arranged in nodules. Fat was abundant, and concentrated mainly in the periphery of the pseudolobules. Slight bile duct proliferation was already noted in the portal tracts.

4. *Carbon tetrachloride for 16 weeks* (fig. 4).

The livers were in a state of advanced cirrhosis. The normal architecture was completely destroyed. Marked connective tissue proliferation and nodular regeneration of parenchymal cells had produced a great number of pseudolobules which varied in size, but were generally smaller than in the 8-week group. Cellular necroses, mitoses and multinuclear cells were seen in great numbers. There was less fat than in the 8-week group. Proliferating connective tissue rich in cells was now also noted in the portal tracts, and the proliferation of bile ducts was abundant. With regard to the degeneration of parenchymal cells, this group differed from the preceding one in that it showed a great number of eosinophilic degeneration of the cells (Councilman-bodies) in addition to the ballooning type of cell degeneration. Furthermore, recent haemorrhages and a relatively large number of macrophages containing haemosiderin were seen.

Summary of morphological changes.

Chronic administration of carbon tetrachloride induced a progressive change in the rat liver. The initial stage of the change was characterized by degeneration and necrosis of the parenchymal cells, beginning in the centrolobular areas and around the collecting veins. This was later followed by nodular regeneration of the parenchymal cells and connective tissue proliferation. Fibrosis had also begun primarily in the centrolobular area. After this fibrous bands developed with united the central veins with each other and with the collecting veins. After only 4 weeks of carbon tetrachloride treatment the histological picture satisfied the first four criteria of cirrhosis. The portal tracts remained intact for a relatively long time but after 8 weeks there appeared changes related to the fifth criterion: i. e. connective tissue

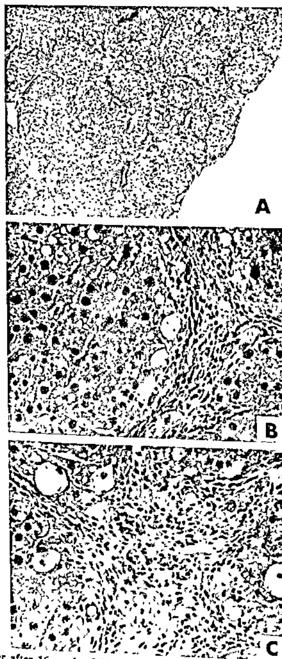


Fig. 4. Rat liver after 16 weeks CCl_4 administration. Stain: van Gieson. A. Complete loss of architecture. Severe cirrhosis. Magnification × 40. B. A thick cellular band between the pseudolobules. Magnification × 250. C. Marked bile duct proliferation in a fibrotic portal tract. Magnification × 250.

3. *Carbon tetrachloride for 8 weeks (fig. 3).*

The changes satisfied the above mentioned criteria of cirrhosis. Thick connective tissue septa rich in fibroblasts united the central veins with each other and with the collecting veins; this created pseudolobules which often had a portal tract in the middle. In some places it was already possible to discern fibrous bands uniting the portal tracts and fibrotic centrolobular areas. In addition to fibroblasts, granulocytes and a few pigmented macrophages were also seen in the fibrotic areas. The pigment was haemosiderin. Necroses of individual cells and mitoses were numerous. The sizes of cells and nuclei varied considerably, and a great number of binuclear cells were seen. No regular liver cell columns were found, the parenchymal cells being arranged in nodules. Fat was abundant, and concentrated mainly in the periphery of the pseudolobules. Slight bile duct proliferation was already noted in the portal tracts.

4. *Carbon tetrachloride for 16 weeks* (fig. 4).

The livers were in a state of advanced cirrhosis. The normal architecture was completely destroyed. Marked connective tissue proliferation and nodular regeneration of parenchymal cells had produced a great number of pseudolobules which varied in size, but were generally smaller than in the 8-week group. Cellular necroses, mitoses and multinuclear cells were seen in great numbers. There was less fat than in the 8-week group. Proliferating connective tissue rich in cells was now also noted in the portal tracts, and the proliferation of bile ducts was abundant. With regard to the degeneration of parenchymal cells, this group differed from the preceding one in that it showed a great number of eosinophilic degeneration of the cells (Councilman-bodies) in addition to the ballooning type of cell degeneration. Furthermore, recent haemorrhages and a relatively large number of macrophages containing haemosiderin were seen.

Summary of morphological changes.

Chronic administration of carbon tetrachloride induced a progressive change in the rat liver. The initial stage of the change was characterized by degeneration and necrosis of the parenchymal cells, beginning in the centrolobular areas and around the collecting veins. This was later followed by nodular regeneration of the parenchymal cells and connective tissue proliferation. Fibrosis had also begun primarily in the centrolobular area. After this fibrous bands developed with united the central veins with each other and with the collecting veins. After only 4 weeks of carbon tetrachloride treatment the histological picture satisfied the first four criteria of cirrhosis. The portal tracts remained intact for a relatively long time but after 8 weeks there appeared changes related to the fifth criterion: *i. e.* connective tissue

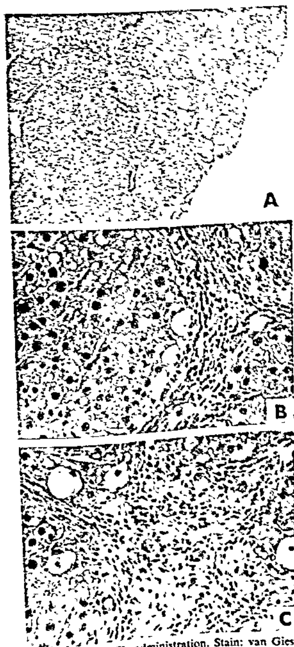


Fig. 4. Rat liver after 16 weeks CCl_4 administration. Stain: van Gieson. A. Complete loss of architecture. Severe cirrhosis. Magnification × 40. B. A thick cellular band between the pseudolobules. Magnification × 250. C. Marked bile duct proliferation in a fibrotic portal tract. Magnification × 250.

Table 2.

Effect of chronic CCl_4 administration on basal serum bilirubin, bilirubin overload test and hexobarbital sleeping time. Serum bilirubin and hexobarbital sleeping time were measured in 6 animals in each group. Bilirubin overload test was carried out on 5 animals in each group. Results are expressed as mean ± S. D.

Duration of treatment	Basal serum bilirubin (mg/100 ml)	Serum bilirubin in bilirubin overload test (mg/100 ml)	% of control	Sleeping time (minutes)	% of control
2 weeks					
control	0.55 ± 0.17	1.88 ± 0.75	100	20 ± 3	100
CCl_4	0.49 ± 0.14	1.75 ± 0.86	93	41 ± 15[b)]	205
4 weeks					
control	0.58 ± 0.20	2.02 ± 0.49	100	19 ± 4	100
CCl_4	0.51 ± 0.17	1.88 ± 0.27	93	47 ± 12[c)]	247
8 weeks					
control	0.55 ± 0.16	1.88 ± 0.61	100	20 ± 4	100
CCl_4	0.58 ± 0.15	3.30 ± 1.73[a)]	176	65 ± 34[b)]	325
16 weeks					
control	0.59 ± 0.15	1.98 ± 0.32	100	18 ± 5	100
CCl_4	0.64 ± 0.10	3.85 ± 1.13[b)]	194	182 ± 59[c)]	1011

a) $P < 0.05$.
b) $P < 0.01$.
c) $P < 0.001$.

trabeculae between the portal tracts and the central veins. After 16 weeks of carbon tetrachloride administration severe cirrhosis was noted. The normal architecture was completely destroyed, connective tissue proliferation was marked, and abundant bile duct proliferation was seen. Changes suggesting biliary stasis could not be observed histopathologically.

Metabolic capacity.

1. Total serum bilirubin and "bilirubin overload test".

Total serum bilirubin concentration in the rats receiving carbon tetrachloride did not differ from the controls at any stage of the 16-week carbon tetrachloride administration period (table 2).

When the test animals were given intravenous injections of unconjugated bilirubin, retention was not increased significantly as compared with the control animals after 2 and 4 weeks of carbon tetrachloride administration. However in the rats which had received carbon tetrachloride for 8 weeks,

bilirubin retention was 76 % greater than in the controls, and after 16 weeks of administration the retention was almost doubled (table 2).

2. Duration of action of hexobarbital.

After carbon tetrachloride administration for 2 weeks hexobarbital sleeping time was approximately twice as long as in the controls. The sleeping time became progressively longer: after 4 weeks of administration it was 2½ times, after 8 weeks more than 3 times, and after 16 weeks approximately 10 times as long as in the controls (table 2).

Discussion

Our finding that chronic administration of carbon tetrachloride induced retarded growth and increased the relative weight of the liver agrees with the results of previous investigations (ISLAMI *et al.* 1958; NEUKOMM & KIRALY 1967; REUBER 1968). It has been indicated earlier that the weight of the left and the median lobes of the rat liver accounts for 70 % (65–75 %) of the total liver weight (HIGGINS & ANDERSON 1931). It has been indicated previously that the proportion of the part thus resected to the whole liver does not differ in cirrhotic and normal rats (RABINOVICI & WIENER 1961), which suggests that the relative weights of the whole livers were greater in the rats which had received carbon tetrachloride than in the controls.

The histopathological changes induced in the rat liver by chronic carbon tetrachloride treatment have been described in several studies (CAMERON & KARUNARATNE 1936; KAUFMANN 1953; ISLAMI *et al.* 1958; WONG & FINCKH 1965; OLSSON 1966). KAUFMANN noted that the initial stage was characterized by degeneration and necrosis of the parenchymal cells, beginning in the centrolobular areas and around the central veins. Parenchymal damage, connective tissue proliferation and the regeneration of parenchymal cells resulted in cirrhosis, which KAUFMANN called hypertrophic cirrhosis. Fibrosis had also begun in the centrolobular area and the portal tracts only became involved at a relatively late period. In his investigation in 1966, OLSSON concentrated on the development of fibrosis and reported that fibrosis began primarily in the wall of the central vein. After that fibrous bands appeared which were oriented towards the periphery as well as fibrous bands which connected the central veins with each other. Portal tract fibrosis began later, but within four months it was so pronounced that it could no longer be distinguished from the centrolobular fibrosis. The morphological changes noted in our study agree with the earlier results. The features described by KAUFMANN (1953) and OLSSON (1966) were particularly clearly obse[illegible] as the cirrhosis progressed.

As early as 4 weeks after the beginning of carbon tetrachloride treatment the histological picture satisfied the first four of the criteria of cirrhosis we used, but it was not until 8 weeks that the changes associated with the fifth criterion appeared: fibrous bands uniting the central veins and the portal tracts. Earlier experiments with dye injections have indicated that these connective tissue bands contain a large number of anastomoses between the portal veins and branches of the hepatic vein, which shunt most of the blood past the parenchymal cells (ASHBURN *et al.* 1947; POPPER *et al.* 1952). Circulatory changes of this kind could be expected to lead to considerable metabolic changes.

It is interesting to note that it was just at the eight week that an increase in the retention of exogenous bilirubin occurred in our experiments. This could be explained partly by the above mentioned changes in the vascular pattern of the liver. It is possible, on the other hand, that microsomal glucuronyl transferase activity had declined. The latter assumption is supported by the earlier observation that bilirubin conjugation is retarded *in vitro* during chronic administration of carbon tetrachloride (METGE *et al.* 1964). An observation made in our laboratory, that microsomal conjugation of p-nitrophenol *in vitro* is similarly retarded after eight weeks of carbon tetrachloride administration, supports the assumption that a decrease in the microsomal conjugational ability is involved (VORNE & ARVELA 1971). Although the bilirubin overload test showed that the retention increased simultaneously with the appearance of cirrhosis, the serum bilirubin basal values did not differ from the control values in any of the groups.

Even after two weeks the duration of action of hexobarbital was significantly longer in the animals receiving carbon tetrachloride than in the controls, and this increased progressively as the morphological liver changes grew more severe. Since the hexobarbital sleeping time increased even before the above mentioned vascular changes had occurred, it seems likely that the increase is mainly due to a decrease in the activity of microsomal enzymes hydroxylating hexobarbital. This assumption is supported by our findings in which we have shown that microsomal N-methylaniline demethylation decreases *in vitro* even during the early stages of liver damage (VORNE *et al.* 1970), and that hexobarbital hydroxylation decreases after only two weeks of carbon tetrachloride administration, after which it decreases progressively (VORNE & ARVELA 1971).

Our *in vivo* experiments show that microsomal drug metabolism is impaired in liver cirrhosis. Our observations agree with the clinical study which showed that the half-life of phenylbutazone, which is metabolized by liver microsomes, was significantly longer in patients with liver disease than in the controls (LEVI *et al.* 1968).

Our results justify the assumption that different microsomal enzymes are

possibly affected in different ways, as the liver damage grows more severe and cirrhosis advances. This could be due to their different location in the endoplasmic reticulum and to the different rates at which parts of the endoplasmic reticulum are damaged. Electron microscopic studies would be necessary to elucidate this point. It is known, however, that glucuronyl transferase activity is mainly located in the rough endoplasmic reticulum, while many of the drug-metabolizing enzymes are found in the smooth endoplasmic reticulum (FOUTS & GRAM 1969).

It is well-known that drug metabolism is different in different species (CREAVEN *et al.* 1965; PARKE 1968), and therefore no direct conclusions concerning human liver cirrhosis can be drawn. It is possible, however, that in the human liver cirrhosis too, different microsomal enzymes are damaged in different ways.

REFERENCES

Ashburn, L. L., K. M. Endicott, F. S. Daft & R. D. Lillie: The non-portal distribution of the trabeculae in dietary cirrhosis of rats and carbon tetrachloride cirrhosis of rats and guinea pigs. *Amer. J. Path. 1947*, **23**, 159–171.

Beck, K., H. A. Kühn & B. Nouraie: Zur Frage der Glucuronidbildung bei toxischen Schädigungen der Leber. *Acta hepato-splenol.* 1967, **14**, 280–291.

Brodie, B. B., J. J. Burns & M. Weiner: Metabolism of drugs in subjects with Laennec's cirrhosis. *Med. exp.* 1959, **1**, 290–292.

Bush, M. T.: Sedatives and hypnotics. 1. Absorption, fate, and excretion. In: *Physiological pharmacology* Vol. 1, p. 199. Ed.: W. S. Root and F. G Hofmann, Academic Press, New York 1963.

Cameron, G. R. & W. A. E. Karunaratne: Carbon tetrachloride cirrhosis in relation to liver regeneration. *J. Path. Bact.* 1936, **42**, 1–21.

Cooper, J. R. & B. B. Brodie: The enzymatic metabolism of hexobarbital (evipal). *J. Pharmacol. exp. Ther.* 1955, *114*, 409–417.

Creaven, P. J., D. V. Parke & R. T. Williams: A fluorimetric study of the hydroxylation of biphenyl in vitro by liver preparations of various species. *Biochem. J.* 1965, **96**, 879–897.

Fouts, J. R. & T. E. Gram: The metabolism of drugs by subfractions of hepatic microsomes: The case for microsomal heterogeneity. In: *Microsomes and drug oxidations*, pp. 81–91. Ed.: J. R Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts & G. J. Mannering, Academic Press, New York 1969.

Higgins, G. M. & R. M. Anderson: Experimental pathology of the liver. I. Restoration of the liver of white rat following partial surgical removal. *Arch. Path.* 1931, **12**, 186–202.

Islami, A. H., G. T. Pack & J. C. Hubbard: Regenerative hyperplasia of the cirrhotic liver following partial hepatectomy. *Cancer* 1958, **11**, 663–686.

Kaltiala, E. H.: The experimental obstructive jaundice and liver microsomal enzymes in rats. *Ann. Chir. Gynaec. Fenn.* 1971, **60**, 43–48.

carbon tetrachloride on the drug-metabolizing capacity of the liver has been mainly investigated after acute administration.* It has been noted, however, that chronic administration of carbon tetrachloride leads to liver damage which gradually becomes more severe and finally ends in cirrhosis (CAMERON & KARUNARATNE 1936; KAUFMANN 1953; OLSSON 1966). So far, very little is known about the drug-metabolizing capacity of the cirrhotic liver. A few clinical investigations have been made, but their results are somewhat contradictory (BRODIE *et al.* 1959; LEVI *et al.* 1968). Experimental liver cirrhosis has been noted to involve decreased glucuronyl transferase activity *in vitro* (METGE *et al.* 1964) and impaired bilirubin and hexobarbital metabolism *in vivo* (VORNE & ALAVAIKKO 1971).

We have investigated the effect of chronic carbon tetrachloride administration on the enzymatic activity of the liver in order to ascertain whether the severity of the liver damage is correlated to its enzymatic activity. We have previously presented the results of our *in vivo* experiments and described the morphological changes which occurred (VORNE & ALAVAIKKO 1971). In the present study we have determined the activity of the drug-metabolizing liver enzymes which oxidize, reduce and conjugate foreign substances, by using hexobarbital, N-methylaniline, *p*-nitrobenzoic acid, and *p*-nitrophenol as substrates. In addition, we have determined the microsomal cytochrome P-450 content of the liver and the activity of non-microsomal glucose-6-phosphate dehydrogenase during the different stages of developing liver damage.

A preliminary report of this work has been published (VORNE *et al.* 1970).

Material and Methods

Treatment of animals.

The present study belongs to a series of experiments in which we used altogether 660 Sprague-Dawley rats which weighed 90–160 g at the beginning of the experiment. They all came from the same farm (The Mankkaa Research Laboratory of Medical Suppliers, Orion Oy, Finland). The animals were given normal laboratory food (The Hankkija Fodder Mixture, Hankkija Central Cooperative, Finland) and water *ad libitum* throughout the experiment. The rats were given 2 ml carbon tetrachloride per kg body weight subcutaneously 3 times a week. The control animals received a corresponding physiological saline solution. After 2, 4, 8, and 16 weeks of dosage we determined the activities of some liver enzymes which metabolized foreign substances, and the cytochrome P-450 content. These examinations were carried out 2 days after the last dose.

* When our study was in preparation MARSHALL & McLEAN (1969) reported that hepatic microsomal cytochrome P-450 content declined during the production of cirrhosis by a combination of carbon tetrachloride and phenobarbital administration.

possibly affected in different ways, as the liver damage grows more severe and cirrhosis advances. This could be due to their different location in the endoplasmic reticulum and to the different rates at which parts of the endoplasmic reticulum are damaged. Electron microscopic studies would be necessary to elucidate this point. It is known, however, that glucuronyl transferase activity is mainly located in the rough endoplasmic reticulum, while many of the drug-metabolizing enzymes are found in the smooth endoplasmic reticulum (FOUTS & GRAM 1969).

It is well-known that drug metabolism is different in different species (CREAVEN *et al.* 1965; PARKE 1968), and therefore no direct conclusions concerning human liver cirrhosis can be drawn. It is possible, however, that in the human liver cirrhosis too, different microsomal enzymes are damaged in different ways.

REFERENCES

Ashburn, L. L., K. M. Endicott, F. S. Daft & R. D. Lillie: The non-portal distribution of the trabeculae in dietary cirrhosis of rats and carbon tetrachloride cirrhosis of rats and guinea pigs. *Amer. J. Path. 1947*, **23**, 159–171.

Beck, K., H. A. Kühn & B. Nouraie: Zur Frage der Glucuronidbildung bei toxischen Schädigungen der Leber. *Acta hepato-splenol.* 1967, **14**, 280–291.

Brodie, B. B., J. J. Burns & M. Weiner: Metabolism of drugs in subjects with Laennec's cirrhosis. *Med. exp.* 1959, **1**, 290–292.

Bush, M. T.: Sedatives and hypnotics. 1. Absorption, fate, and excretion. In: *Physiological pharmacology*. Vol. 1, p. 199. Ed.: W. S. Root and F. G. Hofmann, Academic Press, New York 1963.

Cameron, G. R. & W. A. E. Karunaratne: Carbon tetrachloride cirrhosis in relation to liver regeneration. *J. Path. Bact.* 1936, **42**, 1–21.

Cooper, J. R. & B. B. Brodie: The enzymatic metabolism of hexobarbital (evipal). *J. Pharmacol. exp. Ther.* 1955, *114*, 409–417.

Creaven, P. J., D. V. Parke & R. T. Williams: A fluorimetric study of the hydroxylation of biphenyl in vitro by liver preparations of various species. *Biochem. J.* 1965, *96*, 879–897.

Fouts, J. R. & T. E. Gram: The metabolism of drugs by subfractions of hepatic microsomes: The case for microsomal heterogeneity. In: *Microsomes and drug oxidations*, pp. 81–91. Ed.: J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts & G. J. Mannering, Academic Press, New York 1969.

Higgins, G. M. & R. M. Anderson: Experimental pathology of the liver. I. Restoration of the liver of white rat following partial surgical removal. *Arch. Path.* 1931, **12**, 186–202.

Islami, A. H., G. T. Pack & J. C. Hubbard: Regenerative hyperplasia of the cirrhotic liver following partial hepatectomy. *Cancer* 1958, **11**, 663–686.

Kaltiala, E. H.: The experimental obstructive jaundice and liver microsomal enzymes in rats. *Ann. Chir. Gynaec. Fenn.* 1971, **60**, 43–48

Kaufmann, G.: Tetrachlorkohlenstoff-Zirrhose und Restauration der Leber. *Beitr. path. Anat.* 1953, *113*, 253–270.

Krueger, H. & J. Higginson: The bilirubin overload test with special reference to liver damage produced by India ink. *Proc. Soc. exp. Biol.* (N. Y.) 1961, **107**, 43–47.

Levi, A. J., S. Sherlock & D. Walker: Phenylbutazone and isoniazid metabolism in patients with liver disease in relation to previous drug therapy. *Lancet* 1968, **1**, 1275–1279.

McLuen, E. F. & J. R. Fouts: The effect of obstructive jaundice on drug metabolism in rabbits. *J. Pharmacol. exp. Therap.* 1961, **131**, 7–11.

Metge, W. R., C. A. Owen Jr., W. T. Foulk & H. N. Hoffman: Bilirubin glucuronyl transferase activity in liver disease. *J. Lab clin. Med.* 1964, **64**, 89–98.

Neukomm, S. & M. Kiraly: Studies on the prevention and cure of experimentally induced functional and morphological liver changes. *Arzneimittel-Forsch.* 1967, **17**, 1135–1142.

Olsson, R.: Partial hepatectomy in experimental carbon tetrachloride induced liver fibrosis. *Acta chir. scand.* 1966, suppl. 366.

Parke, D. V.: Factors which affect the metabolism of drugs. In: *Recent advances in pharmacology*, pp. 75–98. Ed.: J. M. Robson & R. S. Stacey. J. & A. Churchill ltd, London 1968.

Popper, H., H. Elias & D. E. Petty: Vascular pattern of the cirrhotic liver. *Amer. J. clin. Path.* 1952, **22**, 717–729.

Rabinovici, N. & E. Wiener: Liver regeneration after partial hepatectomy in carbon tetrachloride-induced cirrhosis in the rat. *Gastroenterology* 1961, **40**, 416–422.

Reuber, M. D.: Carbon tetrachloride induced cirrhosis. Effect of age and sex. *Arch. Path.* 1968, **85**, 275–279.

Seligson, D.: Biochemical considerations of the liver. In: *Diseases of the liver*, pp. 56–100. Ed.: L. Schiff. Pitman Medical Publishing Co., ltd, London 1963.

White, A. E.: The distribution of glucuronyl transferase in cell membranes. In: *Bilirubin metabolism*, pp. 183–188. Ed.: I. A. D. Bouchier & B. H. Billing. Blackwell Scientific Publications, Oxford 1967.

White, D., G. A. Haidar & J. G. Reinhold: Spectrophotometric measurement of bilirubin concentrations in serum of the newborn by the use of microcapillary method *Clin. Chem.* 1958, **4**, 211–222.

Wong, J. & E. S. Finckh: Tolerance to carbon tetrachloride in carbon tetrachloride-induced cirrhosis. *J. Path. Bact.* 1965, **89**, 611–617.

Vorne, M. & P. Arvela: Effect of carbon tetrachloride induced progressive liver damage on drug-metabolizing enzymes and cytochrome P-450 in rat liver. *Acta pharmacol et toxicol.* 1971, **29**, 417–427.

Vorne, M., P. Arvela & N. T. Kärki: The effect of long-term carbon tetrachloride administration on hepatic enzymes in rat. *Scand. J. clin. Lab. Invest.* 1970, **25**, suppl. 113, 74.

Acta pharmacol. et toxicol. 1971, 29, 417–427.

From the Department of Pharmacology, University of Oulu, Finland

Effect of Carbon Tetrachloride Induced Progressive Liver Damage on Drug-Metabolizing Enzymes and Cytochrome P-450 in Rat Liver

By

Martti Vorne and Pentti Arvela

(Received November 9, 1970)

Abstract. Rats were given 2 ml carbon tetrachloride per kg body weight subcutaneously 3 times a week. After 2, 4, 8, and 16 weeks the activities of drug-metabolizing enzymes and glucose-6-phosphate dehydrogenase and cytochrome P-450 content of liver microsomes were determined. Demethylation of N-methylaniline decreased progressively, and after 16 weeks of carbon tetrachloride administration it was 25 % of the control activity Hydroxylation of hexobarbital also decreased progressively and after 16 weeks it was too low to be measured. Conjugation of *p*-nitrophenol did not decrease until 8 weeks of carbon tetrachloride administration, when the animals had developed liver cirrhosis. As cirrhosis became more severe, there was only a slight further decrease in conjugative activity, and after 16 weeks, 40 % of the conjugative activity was still recorded. Nitroreductase activity remained near the control level for the first 8 weeks, but after 16 weeks it was only 36 % of the activity of corresponding control animals. Cytochrome P-450 content decreased progressively, and after 16 weeks it was too low to be measured. Glucose-6-phosphate dehydrogenase activity remained 2–3 times as great as in the controls throughout the 16-week administration period.

Key-words: Carbon tetrachloride – liver damage – drug metabolism – cytochrome P-450.

It is well known that acute carbon tetrachloride administration leads to severe liver damage which is manifested as dissolution of the endoplasmic reticulum and a reduction in the activity of drug-metabolizing enzymes (Recknagel 1967; Dingell & Heimberg 1968). A single dose of carbon tetrachloride administered by gastric intubation has been found to decrease microsomal glucose-6-phosphatase activity by 30 % after 2 hours, and by 59 % 4 hours after administration (Recknagel & Lombardi 1961). It has further been noted that acute administration of carbon tetrachloride impairs the metabolism of aminopyrine (aminophenazonum NF[illegible] [illegible]bital (enhexymalum NFN), and ethylmorphine (Dingell & [illegible] decreases the amount of cytochrome P-450 (Sasame [illegible]

carbon tetrachloride on the drug-metabolizing capacity of the liver has been mainly investigated after acute administration.* It has been noted, however, that chronic administration of carbon tetrachloride leads to liver damage which gradually becomes more severe and finally ends in cirrhosis (CAMERON & KARUNARATNE 1936; KAUFMANN 1953; OLSSON 1966). So far, very little is known about the drug-metabolizing capacity of the cirrhotic liver. A few clinical investigations have been made, but their results are somewhat contradictory (BRODIE *et al.* 1959; LEVI *et al.* 1968). Experimental liver cirrhosis has been noted to involve decreased glucuronyl transferase activity *in vitro* (METGE *et al.* 1964) and impaired bilirubin and hexobarbital metabolism *in vivo* (VORNE & ALAVAIKKO 1971).

We have investigated the effect of chronic carbon tetrachloride administration on the enzymatic activity of the liver in order to ascertain whether the severity of the liver damage is correlated to its enzymatic activity. We have previously presented the results of our *in vivo* experiments and described the morphological changes which occurred (VORNE & ALAVAIKKO 1971). In the present study we have determined the activity of the drug-metabolizing liver enzymes which oxidize, reduce and conjugate foreign substances, by using hexobarbital, N-methylaniline, *p*-nitrobenzoic acid, and *p*-nitrophenol as substrates. In addition, we have determined the microsomal cytochrome P-450 content of the liver and the activity of non-microsomal glucose-6-phosphate dehydrogenase during the different stages of developing liver damage.

A preliminary report of this work has been published (VORNE *et al.* 1970).

Material and Methods

Treatment of animals

The present study belongs to a series of experiments in which we used altogether 660 Sprague-Dawley rats which weighed 90–160 g at the beginning of the experiment. They all came from the same farm (The Mankkaa Research Laboratory of Medical Suppliers, Orion Oy, Finland). The animals were given normal laboratory food (The Hankkija Fodder Mixture, Hankkija Central Cooperative, Finland) and water *ad libitum* throughout the experiment. The rats were given 2 ml carbon tetrachloride per kg body weight subcutaneously 3 times a week. The control animals received a corresponding physiological saline solution. After 2, 4, 8, and 16 weeks of dosage we determined the activities of some liver enzymes which metabolized foreign substances, and the cytochrome P-450 content. These examinations were carried out 2 days after the last dose.

---

* When our study was in preparation MARSHALL & MCLEAN (1969) reported that hepatic microsomal cytochrome P-450 content declined during the production of cirrhosis by a combination of carbon tetrachloride and phenobarbital administration.

Enzyme preparations.

To obtain the liver samples the animals were subjected to approximately 70 % liver resection under light ether anaesthesia, as reported in our previous work (VORNE & ALAVAIKKO 1971). The liver specimens were immediately rinsed with ice-cold phosphate buffer (0.1 M, pH 7.4), and the same phosphate buffer was used in making a 20 % homogenate in a Potter-Elvehjem type of glass homogenizer. The homogenate was centrifuged at 12,000 × g at 4° for 20 minutes in a refrigerated MSE Superspeed 25 centrifuge. Part of the supernatant fraction was centrifuged at 105,000 × g at 4° for one hour in a refrigerated MSE Superspeed 50 ultracentrifuge. The microsomal pellet was suspended in the phosphate buffer in such a way that 1 ml suspension corresponded to 800 mg of liver.

Enzyme assays.

The 12,000 × g supernatant was used as the enzyme preparation in the assay of N-methylaniline demethylation, hexobarbital (enhexymalum NFN) hydroxylation (mainly side-chain oxidation), and *p*-nitrobenzoic acid reduction. In assaying *p*-nitrophenol conjugation and cytochrome P-450, the microsomal suspension was used as the enzyme preparation. Glucose-6-phosphate dehydrogenase activity was measured from the soluble fraction.

In the investigations of the metabolism of N-methylaniline and hexobarbital, the incubation mixture contained 2 ml enzyme preparation, 120 μmol nicotinamide, 10 μmol $MgCl_2$, 200 μmol KCl, 6 μmol glucose-6-phosphate, 0.25 μmol NADP, and sufficient phosphate buffer (0.1 M, pH 7.4) to make a final volume of 4 ml. The incubation was performed at 37° in a metabolic shaker under an atmosphere of air. In assays of *p*-nitrobenzoic acid the conditions were similar except that nicotinamide was omitted, since it was found to inhibit the reaction, and an atmosphere of nitrogen was used instead of air. The amounts of substrate and the incubation times were as follows: N-methylaniline 5 μmol, 30 min., hexobarbital 2 μmol, 15 min., and *p*-nitrobenzoic acid 3 μmol, 60 min. In the investigations of *p*-nitrophenol conjugation, the incubation mixture contained 0.5 ml microsomal suspension, 0.33 μmol UDPGA ammonium salt, and 0.2 μmol substrate. The volume of the incubation mixture was 1 ml, and the 20-minute incubation took place under an atmosphere of air.

The rate of N-methylaniline demethylation was assayed by measuring the aniline formed during incubation (BRODIE & AXELROD 1948) The rate of hexobarbital metabolism was estimated by measuring the disappearance of substrate (COOPER & BRODIE 1955). *p*-Nitrobenzoic acid reduction was assayed by measuring the *p*-aminobenzoic acid formed during incubation (FOUTS & BRODIE 1957), and *p*-nitrophenol conjugation by measuring the disappearance of substrate (POGELL & LELOIR 1961).

Glucose-6-phosphate dehydrogenase activity was assayed by following the reduction of NADP according to LÖHR & WALLER's method (1965). The measurements were made at room temperature.

Cytochrome P-450 content.

Cytochrome P-450 content was measured according to the method described by OMURA & SATO (1964), by using a Unicam SP 800 spectrophotometer with an external recorder. In the assays the protein concentrations were 2–3 mg/ml.

Protein concentration.

Microsomal protein was assayed by the biuret procedure (LAYNE 1957).

In the investigations of demethylation, glucose-6-phosphate dehydrogenase, and cyto-

chrome P-450, the spectrophotometric examinations were performed with a Unicam SP 800 spectrophotometer. In other spectrophotometric measurements a Beckman DU spectrophotometer was used.

Statistics.
Student's t-test was used in calculating the significance of the results.

Results

Body weight and development of cirrhosis.

In our earlier work we reported that the animals which received carbon tetrachloride gained weight more slowly than the controls, and that their growth discontinued after 2 months of administration. The histological specimens revealed that liver damage grew progressively more severe. Cirrhosis was noted after 8 weeks of carbon tetrachloride administration, and after 16 weeks cirrhosis was severe. The livers of the animals receiving carbon tetrachloride enlarged progressively (VORNE & ALAVAIKKO 1971).

Demethylation of N-methylaniline.

N-demethylase activity of the rats receiving carbon tetrachloride continued to decline throughout the experiment. After 2 weeks of administration the activity had fallen by 50 %. Thereafter the decrease of enzyme activity continued more slowly: after 4 weeks of administration the activity was 44 %, after 8 weeks, 32 %, and after 16 weeks, 25 % of the corresponding control activity (table 1, fig. 1).

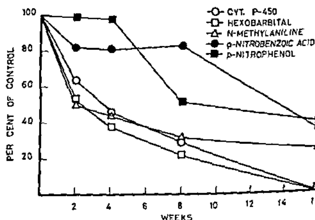


Fig. 1. Summary of effects of chronic CCl_4 treatment on cytochrome P-450 content, hexobarbital hydroxylation, N-methylaniline demethylation, *p*-nitrobenzoic acid reduction and *p*-nitrophenol conjugation in rat liver.

Table 1.

Effect of chronic CCl_4 treatment on demethylation of N-methylaniline, hydroxylation of hexobarbital, reduction of *p*-nitrobenzoic acid and conjugation of *p*-nitrophenol.

Duration of treatment	µMol metabolized per gram liver per hour. Mean ± S. E. M.							
	Demethylation Aniline formed	% of control	Hexobarbital oxidized	% of control	Nitroreduction *p*-Aminobenzoic acid formed	% of control	*p*-Nitrophenol conjugated	% of control
2 weeks								
control	1.53 ± 0.04 (5)	100	6.18 ± 0.38 (6)	100	3.04 ± 0.08 (6)	100	0.170 ± 0.014 (6)	100
CCl_4	0.77 ± 0.04 (5)[c]	50	3.34 ± 0.55 (6)[b]	54	2.50 ± 0.12 (6)[b]	82	0.168 ± 0.016 (6)	99
4 weeks								
control	1.52 ± 0.06 (5)	100	6.11 ± 0.37 (8)	100	2.08 ± 0.04 (8)	100	0.151 ± 0.018 (8)	100
CCl_4	0.67 ± 0.04 (7)[c]	44	2.30 ± 0.23 (8)[c]	38	1.69 ± 0.04 (8)[a]	81	0.148 ± 0.014 (8)	98
8 weeks								
control	1.37 ± 0.08 (6)	100	6.12 ± 0.21 (8)	100	2.30 ± 0.03 (8)	100	0.178 ± 0.008 (8)	100
CCl_4	0.44 ± 0.06 (6)[c]	32	1.36 ± 0.31 (8)[c]	22	1.90 ± 0.06 (8)	83	0.093 ± 0.014 (8)[c]	52
16 weeks								
control	1.19 ± 0.10 (6)	100	4.93 ± 0.31 (8)	100	2.30 ± 0.05 (8)	100	0.152 ± 0.008 (8)	100
CCl_4	0.30 ± 0.03 (8)[c]	25	0 (8)[c]	0	0.83 ± 0.02 (8)[c]	36	0.061 ± 0.011 (8)[c]	40

he number of rats used in brackets.

$P < 0.05$.

$P < 0.01$.

$P < 0.001$.

Hydroxylation of hexobarbital.

Hexobarbital metabolism decreased progressively. After 2 weeks, the hydroxylation activity was 54 %, after 4 weeks, 38 %, and after 8 weeks, 22 % of the corresponding control activity. After 16 weeks hexobarbital metabolism was too low to be measured (table 1, fig. 1).

Reduction of p-nitrobenzoic acid.

p-Nitrobenzoic acid metabolism remained near the control level for the first 8 weeks, but decreased suddenly after 16 weeks of carbon tetrachloride administration, being then only 36 % of the control activity (table 1, fig. 1).

Conjugation of p-nitrophenol.

p-Nitrophenol conjugation remained at the normal level in the animals which had received carbon tetrachloride for 2 and 4 weeks, but after 8 weeks it had fallen to 52 %, and after 16 weeks to 40 % of the corresponding control activity (table 1, fig. 1).

Cytochrome P-450.

The cytochrome P-450 content decreased progressively in the microsomes of the animals which received carbon tetrachloride. After 2 weeks it was 64 %, after 4 weeks, 46 %, and after 8 weeks, 29 % of the corresponding control activity. After 16 weeks, the cytochrome P-450 content was too small to be assayed (table 2, fig. 1).

Microsomal protein.

After 4, 8, and 16 weeks of carbon tetrachloride administration the microsomal protein values were significantly lower than in the controls, but no progressive change in decrease was noted (table 2).

Glucose-6-phosphate dehydrogenase.

All the groups showed glucose-6-phosphate dehydrogenase activity which was 2–3 times as great as that of the controls (table 2).

Discussion

We indicated previously that chronic administration of carbon tetrachloride led to a progressive morphological liver change: after 8 weeks the animals developed liver cirrhosis, which was severe after 16 weeks of administration (VORNE & ALAVAIKKO 1971). Our observations of liver changes agree with other histopathological studies (CAMERON & KARUNARATNE 1936; KAUFMANN 1953; OLSSON 1966).

In the present work it was noted that oxidative microsomal enzyme activi-

Table 2.

Effect of chronic CCl_4 treatment on microsomal protein, microsomal cytochrome P-450 content and glucose-6-phosphate dehydrogenase (G-6-PDH) activity.

Duration of treatment	Microsomal protein mg/g liver	% of control	P-450 μMol/g liver	% of control	G-6-PDH units/g liver	% of control
2 weeks						
control	19.2 ± 1 1 (6)	100	6.71 ± 0.43 (6)	100	12.4 ± 1.1 (5)	100
CCl_4	19.2 ± 0.9 (6)	100	4.31 ± 0.64 (6)[a]	64	37.6 ± 5.4 (5)	303
4 weeks						
control	16.3 ± 0.4 (8)	100	5.98 ± 0.48 (8)	100	17.4 ± 2.3 (5)	100
CCl_4	14.0 ± 0 5 (8)[b]	86	2.73 ± 0.33 (8)[c]	46	39 0 ± 3.3 (7)[c]	224
8 weeks						
control	15.5 ± 0.8 (8)	100	6.98 ± 0.62 (8)	100	8.9 ± 0.6 (6)	100
CCl_4	13 7 ± 1.0 (8)[a]	88	2.00 ± 0.23 (8)[c]	29	27.8 ± 3.0 (6)[c]	312
16 weeks						
control	10 9 ± 0.5 (8)	100	6.91 ± 0.35 (8)	100	11.8 ± 0.7 (6)	100
CCl_4	8.4 ± 0.3 (8)[b]	77	0 (8)[c]	0	35 4 ± 1.8 (8)[c]	300

ıe number of rats used in brackets. Results are expressed as mean of the values ± S. E. M.

[a] < 0.05.

[b] < 0.01.

[c] $P < 0.001$.

ties decreased progressively. N-demethylation activity initially decreased more rapidly than hexobarbital hydroxylation, but at the stage of severe cirrhosis, i. e. after 16 weeks of carbon tetrachloride administration, 25 % of the control demethylation activity was recorded, while the rate of hexobarbital metabolism had become so low that it could no longer be measured.

The decrease in glucuronyl transferase activity was clearly different from the decrease in oxidative enzyme activities. After 4 weeks of carbon tetrachloride administration, conjugative activity still remained at the control level, and only deviated from it after 8 weeks, when cirrhosis was already apparent. As cirrhosis grew more severe, there was only a slight further decrease in conjugative activity.

Nitroreductase activity did not fall notably below the control value until the stage of severe cirrhosis, and in this way it was different from both the oxidative and conjugative activities.

The differences between the changes in oxidative, conjugative and reductive enzyme activities may be due to the different rates at which parts of the endoplasmic reticulum are damaged during chronic carbon tetrachloride administration. To elucidate this point, it would be necessary to perform electron microscopic investigations, and to fractionate microsomes into the smooth and rough endoplasmic reticulum as well as to carry out metabolic investigations on these fractions. It is known that glucuronyl transferase activity is located mainly in the rough endoplasmic reticulum, while NADPH-dependent enzymes are located mainly in the smooth endoplasmic reticulum (FOUTS & GRAM 1969). This could be the reason for the different rate at which conjugative activity changes. Except in the microsomes, nitroreductase activity is also present in the soluble fraction (KATO *et al.* 1969). In our work, nitroreductase was assayed from the 12,000 × g supernatant fraction, and thus both microsomal enzyme activity and enzyme activity of the soluble fraction were measured together. It is possible that the nitroreductase activities of the microsomes and of the soluble fraction change in different ways, which would partly explain the difference noted between the changes in oxidative and reductive enzyme activities.

Microsomal cytochrome P-450 content was found to decrease progressively in the rats which received carbon tetrachloride, and at the stage of severe cirrhosis, after 16 weeks of administration, the content was so small that it could no longer be measured.

It is generally considered that microsomal oxidative reactions are transmitted through cytochrome P-450 (PARKE 1968a). The present results show that oxidative enzyme activities decreased in the same way as the cytochrome P-450 content. It has also been noted previously that there are at least two kinds of cytochrome P-450 which differ from each other in several respects. Among other things, they are affected differently by various inducers, and

they differ in their tolerance to lipase as well as in their stability during storage of the microsomal preparations (MANNERING *et al.* 1969). The differences we noted in the decrease in N-methylaniline demethylation and hexobarbital hydroxylation could be explained by the possibility that their metabolism is transmitted through the different kinds of cytochrome P-450 which are damaged in different ways. In our assays we have measured mainly the cytochrome P-450 which is responsible for hexobarbital hydroxylation and the amount of which decreases in accordance with the decrease in hexobarbital metabolism. However, the decrease in reductive and conjugative activities did not seem to follow the decline in cytochrome P-450. Cytochrome P-450 is apparently not needed in conjugation. It has been noted previously that microsomal nitroreductase activity and cytochrome P-450 content are correlated (GILLETTE & GRAM 1969), and that nitroreductase activity is also correlated with cytochrome P-450 reduction (SASAME & GILLETTE 1969). Our study, however, does not show distinct correlation between nitroreduction and cytochrome P-450. This difference from earlier results can be explained partly by the nitroreductase of the soluble fraction which we also measured, but quantitatively it should not be so great as to explain the difference entirely (KATO *et al.* 1969).

Glucuronyl transferase activity decreases after 8 weeks and correlates well with *in vivo* bilirubin metabolism which also decreased after 8 weeks of carbon tetrachloride administration (VORNE & ALAVAIKKO 1971). The decrease in the metabolism of hexobarbital correlates well with our earlier observation that the duration of action of hexobarbital becomes progressively longer during carbon tetrachloride administration (VORNE & ALAVAIKKO 1971). It therefore seems that the changes we noted previously would, at least to a considerable extent, be due to a decrease in microsomal enzyme activities.

The glucose-6-phosphate dehydrogenase activity of the animals receiving carbon tetrachloride was 2–3 times as great as that of the controls throughout the experiment. It has been noted that the glycogen content of the liver decreases at the same time as the decrease in drug metabolism (FOUTS 1963). It has also been indicated that chronic carbon tetrachloride administration leads to a decrease in the glycogen content of the liver, in which case the synthesis of glycogen is presumably retarded (KANICS & RUBINSTEIN 1968). Changes in glycogen metabolism may lead to increased utilization of glucose through the pentose phosphate cycle, which would explain the rise in glucose-6-phosphate dehydrogenase activity. On the other hand, the maintenance of regenerative processes in the liver increases the need for $NADPH_2$, and the fact that glucose-6-phosphate dehydrogenase is the main producer of $NADPH_2$ could partly explain the increase in glucose-6-phosphate dehydrogenase activity (PLATT & COCKRILL 1969).

Though the biotransformation of drugs varies from one species to another (PARKE 1968b), the drug-metabolizing enzymes are generally located in the liver microsomes and our studies suggest that severe liver diseases may also lead to impairment of drug metabolism in humans. This assumption is supported by human studies in which a prolongation of the half-lives of chloramphenicol (KUNIN *et al.* 1959) and phenylbutazone (LEVI *et al.* 1968) was found in patients with cirrhosis or other liver diseases.

REFERENCES

Brodie, B. B. & J. Axelrod: The estimation of acetanilide and its metabolite products, N-acetyl *p*-aminophenol and *p*-aminophenol (free and total conjugated) in biological fluids and tissues. *J. Pharmacol. exp. Therap.* 1948, **94**, 22–28.

Brodie, B. B., J. J. Burns & M. Weiner: Metabolism of drugs in subjects with Laennec's cirrhosis. *Med. exp.* (Basel) 1959, **1**, 290–292.

Cameron, G. R. & W. A. E. Karunaratne: Carbon tetrachloride cirrhosis in relation to liver regeneration. *J. Path. Bact.* 1936, **42**, 1–21.

Cooper, J. R. & B. B. Brodie: The enzymatic metabolism of hexobarbital (evipal). *J. Pharmacol. exp. Therap.* 1955, **114**, 409–417.

Dingell, J. V. & M. Heimberg: The effects of aliphatic halogenated hydrocarbons on hepatic drug metabolism. *Biochem. Pharmacol.* 1968, **17**, 1269–1278.

Fouts, J. R.: Factors affecting hepatic microsomal enzyme systems involved in drug metabolism. In: *Advances in enzyme regulation*, pp. 225–233. Vol. 1. Ed.: G. Weber. Pergamon Press, London, 1963.

Fouts, J. R. & B. B. Brodie: The enzymatic reduction of chloramphenicol, *p*-nitrobenzoic acid and other aromatic nitro compounds in mammals. *J. Pharmacol. exp. Therap.* 1957, **119**, 197–207.

Fouts, J. R. & T. E. Gram: The metabolism of drugs by subfractions of hepatic microsomes: the case for microsomal heterogenity. In: *Microsomes and drug oxidations*, pp. 81–91. Ed.: J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts & G. J. Mannering. Academic Press, New York 1969.

Gillette, J. R. & T. E. Gram: Cytochrome P-450 reduction in liver microsomes and its relationship to drug metabolism. In: *Microsomes and drug oxidations*, pp. 133–149. Ed.: J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts & G. J. Mannering. Academic Press, New York 1969.

Kanics, L. & D. Rubinstein: The effect of chronic carbon tetrachloride inhalation on the secretion of lipid by rat liver. *Biochem. Pharmacol.* 1968, **17**, 1959–1967.

Kato, R., T. Oshima & A. Takanaka: Studies on the mechanism of nitro reduction by rat liver. *Mol. Pharmacol.* 1969, **5**, 487–498.

Kaufmann, G.: Tetrachlorkohlenstoff-Zirrhose und Restauration der Leber. *Beitr. path. Anat.* 1953, **113**, 253–270.

Kunin, C. M., A. J. Glazko & M. Finland: Persistence of antibiotics in blood of patients with acute renal failure. II Chloramphenicol and its metabolic products in the blood of patients with severe renal disease or hepatic cirrhosis. *J. clin. Invest.* 1959, **38**, 1498–1508.

Layne, E.: Protein estimation with biuret method. In: *Methods in enzymology*, pp. 450–451. Vol. 3. Ed.: S. P. Colowick & N. O. Kaplan. Academic Press Inc., New York 1957.

Levi, A. J., S. Sherlock & D. Walker: Phenylbutazone and isoniazid metabolism in patients with liver disease in relation to previous drug therapy. *Lancet* 1968, **1**, 1275–1279.

Lohr, G. W. & H. D. Waller: Glucose-6-phosphate dehydrogenase. In: *Methods of enzymatic analysis*, pp. 744–751. Ed.: H.-U. Bergmeyer. Academic Press, New York 1965.

Mannering, G. J., N. E. Sladek, C. J. Parli & D. W. Shoeman: Formation of a new P-450 hemoprotein after treatment of rats with polycyclic hydrocarbons. In: *Microsomes and drug oxidations*, pp. 303–330. Ed.: J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts & G. J. Mannering. Academic Press, New York 1969.

Marshall, W. J. & A. E. M. McLean: The effect of cirrhosis of the liver on microsomal detoxications and cytochrome P-450. *Brit. J. exp. Path.* 1969, **50**, 578–583.

Metge, W. R., C. A. Owen Jr., W. T. Foulk & H. N. Hoffman: Bilirubin glucuronyl transferase activity in liver disease. *J. Lab. clin. Med.* 1964, **64**, 89–98.

Olsson, R.: Partial hepatectomy in experimental carbon tetrachloride induced liver fibrosis. *Acta chir. scand.* 1966, Suppl. 366.

Omura, T. & R. Sato: The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. biol Chem.* 1964, **239**, 2370–2378.

Parke, D. V.: The metabolism of drugs. In: *Recent advances in pharmacology*, pp. 29–74. Ed: J. M. Robson & R. S. Stacey, J. & A. Churchill Ltd., London 1968a.

Parke, D. V.: Factors which affect the metabolism of drugs. In: *Recent advances in pharmacology*, pp. 29–74. Ed.: J. M. Robson & R. S. Stacey, J. & A. Churchill Ltd, London 1968b.

Platt, D. S. & B. L. Cockrill: Biochemical changes in rat liver in response to treatment with drugs and other agents – III effects of centraly acting drugs. *Biochem Pharmacol.* 1969, **18**, 459–473.

Pogell, B. M. & L. F. Leloir: Nucleotide activation of liver microsomal glucuronidation. *J. biol Chem.* 1961, **236**, 293–298.

Recknagel, R. O. & B. Lombardi: Studies of biochemical changes in subcellular particles of rat liver and their relationship to a new hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation. *J. biol. Chem.* 1961, **236**, 564–569.

Recknagel, R. O.: Carbon tetrachloride hepatotoxicity. *Pharmacol. Rev.* 1967, **19**, 145–208.

Sasame, H. A., J. A. Castro & J. R. Gillette: Studies on the destruction of liver microsomal cytochrome P-450 by carbon tetrachloride administration. *Biochem. Pharmacol.* 1968, **17**, 1739–1768.

Sasame, H. A. & J. R. Gillette: Studies on the relationship between the effects of various substances on absorption spectrum of cytochrome P-450 and the reduction of p-nitrobenzoate by mouse liver microsomes *Molec. Pharmacol.* 1969, **5**, 123–130.

Vorne, M. & M. Alavaikko: Effect of carbon tetrachloride induced progressive liver damage on the metabolism of hexobarbital and bilirubin *in vivo*. *Acta pharmacol. et toxicol.* 1971, **29**, 402–416.

Vorne, M., P. Arvela & N. T. Karki: The effect of long-term carbon tetrachloride administration on hepatic enzymes in rat. *Scand. J. clin Lab. Invest.* 1970, **25**, suppl. 113, 74.

Acta pharmacol. et toxicol. 1971, 29, 428–440.

From the Department of Pharmacology, University of Copenhagen, Denmark
(Professor Jens Schou, M. D.)

Renal Excretion of Riboflavin in the Rat

By

Sten Christensen

(Received October 14, 1970)

Abstract: Renal clearances of riboflavin and inulin-^{14}C-carboxylic acid were determined in Wistar rats anaesthetized with ethanol + N_2O or amobarbital sodium. At plasma riboflavin concentrations ranging from 0.5 to 10 µg/ml the riboflavin:inulin clearance ratio varied from 1.2 to 3.2, indicating active tubular secretion of riboflavin. Probenecid, 10–75 mg/kg intra-arterially, depressed the clearance ratio to approximately 1.0. Since the binding of riboflavin to proteins averaged 21 %, this indicates that back diffusion does not occur tubules. Precipitation of riboflavin in the kidneys at high plasma levels pr the demonstration of a possible tubular maximum (Tm) for the tubular se The clearance ratio was generally unaffected by considerable variations glomerular filtration rate (GFR). At very low plasma levels, howev clearance ratio varied proportionally with the GFR and the urine flow. on this basis it is suggested that riboflavin, as well as being secreted i tubules, is in addition reabsorped from the tubules by an easily s mechanism.

Key-words: Riboflavin – renal excretion.

Previous studies in rats (Christensen 1969b) have suggested flavin is eliminated partly by renal tubular secretion. The purp study has been to elucidate further the renal excretion of this the rat.

Methods

Female Wistar rats, 250–310 g, on a standard diet, were used

Plasma protein binding.

The binding of riboflavin to plasma proteins was determined *in vitro* by the ultrafiltration technique of Ames & Sakanoue (1964). Riboflavin-2-^{14}C (The radiochemical Centre, Amersham, England), 0.25–14 µg/ml, was added to heparinized blood plasma pooled from 10 rats. After equilibration for 1 hour at 37° *in an atmosphere of* 96 % O_2 and 4 % CO_2 a plasma ultrafiltrate was *obtained through a* Viskinge® dialysis tubing with an average pore diameter of 24 A. Approximately 0.5 ml of the ultrafiltrate was

collected from 2.5 ml of plasma, by applying a pressure of 1 kg/cm². Corrections for the binding of riboflavin to the membrane were obtained by ultrafiltration of isotonic saline containing the same concentrations of riboflavin.

Animal technique.

Anaesthesia was induced with 4 ml 10 % (v/v) ethanol/100 g body weight given orally, supplemented by inhalation of $N_2O + O_2$ (4:1), or with amobarbital (pentymalum NFN) sodium, 10 mg/100 g body weight, intraperitoneally. The rat was fixed in a supine position on a heated surgical table, and the rectal temperature kept between 37 and 38°. A tracheal canulla was inserted and, in experiments with inhalation anaesthesia, connected to an anaesthesia apparatus. A heparinized polyethylene tube was inserted into one of the carotid arteries and connected to a membrane transducer for registration of the arterial blood pressure. The urinary bladder was made accessible by a small incision through the abdominal wall, and a polyethylene catheter was inserted through a puncture made at the end opposite to the urethra. The bladder was ligated around the catheter which was kept in a position that allowed a free urine flow. Infusions were performed through the femoral veins.

Clearance technique.

Riboflavin (Sigma, St. Louis, Miss., U. S. A.) and carrier free inulin-^{14}C-carboxylic acid (NEN, Boston, Mass., U. S. A.), usually contained in the same isotonic solution, were infused at a constant rate, the infusion volume being 0.5 or 1.0 ml/hour. In experiments designed to obtain different plasma levels of riboflavin, the latter was infused separately in different successive concentrations, the infusion volume rate being constant. A dextran-glucose solution (Infudex®, containing 6 % dextran, mol.wt. 55.000 and 5 % glucose) was used as a substitute for the drawn blood samples, either infused continously or given as single injections. Arterial blood samples, 150–300 µl each, were drawn every 15 min. Urine was sampled 3 min. later, the bladder being washed with 0.4 ml of isotonic saline. Basal flavin contents in the blood and urine were subtracted before calculation of the riboflavin clearance. The net tubular transport rate (T) was calculated from the formula:

$$T = U - GFR \cdot P \cdot F \cdot W = U - GFR \cdot P \cdot 0.79$$

where U = the urinary excretion rate, GFR = the glomerular filtration rate, P = the plasma concentration of riboflavin, F = the unbound fraction in the plasma i.e. the ratio of the concentration in the ultrafiltrate to that in whole plasma (0.85) and W = the fraction of plasma which is water (0.93).

Dosages.

Carrier free inulin-^{14}C-carboxylic acid (specific activity 1.84 mci/g) was infused at a rate of 0.7–1.0 mg/hour. The infusion rate of riboflavin ranged from 0.02 to 22 mg/hour. Highly concentrated solutions of riboflavin were prepared by dissolving it in diluted sodium hydroxide at 80° and, after cooling, readjusting the pH to 9.5 with hydrochloric acid. In three experiments probenecid (Doldor, Germany), 40, 80 or 300 mg/kg body weight, was injected intra-arterially. The drug was dissolved in diluted sodium hydroxide and the pH readjusted to 9.5 with hydrochloric acid.

Analytical techniques.

Riboflavin was assayed fluorometrically by the method of Burch *et al.* (1948) as modified by Christensen (1969a) using a "Photovolt" fluorometer. The radioact[illegible] of

the labelled compounds was determined by liquid scintillation counting in a Packard *Tri-carb spectrometer model 3380*, using BRAY'S (1960) scintillation medium.

Fifty–100 μl of plasma was added to 1.5–2.5 ml of 5 % trichloracetic acid (TCA). Urine samples were diluted with water up to 5.00 ml, and 100–500 μl of the diluted urine was added to 1.5–2.5 ml of 5 % TCA. After centrifugation 0.5–1.0 ml TCA extracts of plasma or urine were added to 10 ml of the scintillation medium. The radioactivity was determined with a maximum counting error of 1.0 %. Since the degree of quenching (as seen from the external standard ratio) was generally constant and the same in the plasma and urine vials, the amounts of inulin-^{14}C-carboxylic acid could be calculated in terms of cpm. For the assay of riboflavin, the TCA extracts of plasma and urine were neutralized to pH 6.5 with 0.5 M-K_2HPO_4 and the fluorescence measured in the manner described above.

Results

Plasma protein binding.

The binding *in vitro* of riboflavin to plasma proteins is shown in fig. 1. Each point represents the mean of duplicate assays carried out on the same pooled plasma. The fraction bound is 21 ± 3 % (S. D.), independantly of

Table 1.

Plasma riboflavin concentration, inulin clearance and riboflavin:inulin clearance ratio in experiments with *constant* infusion rate. The experiments are arranged according to increasing plasma concentration of riboflavin.

Exp. no.	Infusion rate of riboflavin (mg/hr)	No. of periods	Plasma riboflavin concentration (μg/ml) Mean (Range)	Inulin clearance (GFR) (ml/min.) Mean (Range)	Riboflavin:inulin clearance ratio Mean ± S. E. M.
30	0.02	11	0.08 (0.05–0.10)	1.34 (0.63–2.14)	1.50 ± 0.03
24	0.86	4	0.65 (0.47–0.75)	2.14 (1.88–2.42)	3.08 ± 0.12
5	0.18	6	0.67 (0.48–1.06)	0.81 (0.34–2.01)	2.23 ± 0.12
3	0.34	5	1.00 (0.42–1.28)	1.35 (0.99–2.08)	2.17 ± 0.03
4	0.18	19	1.05 (0.44–2.11)	0.62 (0.15–1.94)	1.47 ± 0.03
2	0.34	6	1.52 (1.32–1.64	0.58 (0.38–1.20)	3.17 ± 0.08
23	0.86	4	1.53 (1.06–2.00)	1.14 (0.89–1.49)	1.33 ± 0.04
22	0.50	12	1.75 (1.65–2.01)	2.44 (2.21–2.73)	1.59 ± 0.01
19	0.94	18	2.47 (1.93–2.96)	1.22 (0.31–3.03)	1.98 ± 0.05
18	1.02	10	2.57 (1.90–3.17)	1.38 (0.88–1.66)	1.90 ± 0.03
14	0.40	5	2.74 (1.33–4.38)	1.06 (0.32–1.47)	2.19 ± 0.04
7	0.92	12	3.07 (2.04–4.86)	1.46 (0.69–2.07)	2.38 ± 0.11
8	1.05	11	5.31 (3.22–8.87)	1.18 (0.35–1.94)	1.83 ± 0.04
1	1.79	5	8.60 (6.73–10.8)	0.50 (0.23–0.89)	3.01 ± 0.13
15	4.2	3	21.00 (18.00–23.00)	1.70 (1.25–1.99)	2.15 ± 0.13

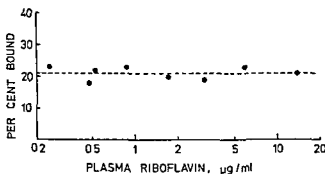


Fig. 1. The *in vitro* binding of riboflavin to plasma proteins at different plasma concentrations. The points represent the means of duplicate assays. The broken line indicates the average binding: 21 ± 3 % (S. D.)

Table 2.

Plasma riboflavin concentration, inulin clearance and riboflavin:inulin clearance ratio in some experiments with constant infusion rate before (control) and after the administration of *probenecid*. The infusion rate of riboflavin was about 1.0 mg/hour. Probenecid was given intra-arterially as single or repeated injections. Cumulated doses were 10 mg (experiment 16), 20 mg (experiment 13) and 75 mg (experiment 10).

Exp. no.		No of periods	Plasma riboflavin concentration (µg/ml) Mean (Range)	Inulin clearance (GFR) (ml/min.) Mean (Range)	Riboflavin: inulin clearance ratio Mean ± S. E. M.
16	Control	3	1.35 (1.14–1.54)	1.58 (1 43–1 63)	2.85 ± 0.08
	Probenecid	7	3.75 (3 36–4 17)	1.80 (1.38–2.39)	1.01 ± 0.01**
13	Control	3	1 86 (1 28–2.65)	1 60 (0.64–2.40)	2.03 ± 0.18*
	Probenecid	5	5.65 (4.19–6.91)	0 82 (0.41–1.05)	0.82 ± 0.02
10	Control	3	2.62 (2.27–2.89)	1.70 (1.61–1.85)	2.56 ± 0 03**
	Probenecid	4	5.83 (4.50–7.61)	1.01 (0.59–2.00)	0.91 ± 0.01

* Significantly lower than the control value at $P < 0.05$.
** Significantly lower than the control value at $P < 0.001$.

the plasma concentration in the range investigated. This binding corresponds to an unbound fraction in the plasma water of 0.85. No binding of inu[illegible] ^{14}C-carboxylic acid was demonstrated.

Table 3.

Plasma riboflavin concentration, inulin clearance and riboflavin:inulin clearance ratio in experiments with *varying* infusion rates.

Exp. no.	Infusion rate of riboflavin (mg/hr)	No. of periods	Plasma riboflavin concentration (μg/ml) Mean (Range)	Inulin clearance (GFR) (ml/min) Mean (Range)	Riboflavin:inulin clearance ratio Mean ± S. E. M.
27	0.04 0.16 0 83	5 4 4	0.07 (0.05–0.09) 0.29 (0.23–0.36) 1.82 (1.59–1.99)	1.27 (1.04–2.00) 1.65 (1.20–1.92) 1.66 (1.58–1.79)	1.27 ± 0 11 2.03 ± 0.03 1 80 ± 0 02
26	0.04 0.17 0.86	5 3 3	0.08 (0.05–0.10) 0.45 (0.42–0.48) 2.19 (2.04–2.38)	1.42 (0 40–2.11) 1.34 (1.03–1.60) 1.41 (1.18–1.70)	1.20 ± 0.08 1.19 ± 0.14 1.22 ± 0 10
25	0.19 0.94 4.67	3 3 3	0.17 (0.16–0.18) 1.01 (0.98–1.07) 9.07 (5.98–12.6)	2.76 (2.52–3.11) 1.58 (1.31–1.85) 1.53 (1.39–2.01)	1.40 ± 0.28 2.20 ± 0 19 2.00 ± 0.16
24	0.86 4.31	4 4	0.65 (0.47–0.75) 8.66 (7.00–9.86)	2.14 (1.88–2.42) 1.22 (1.12–1.36)	3.08 ± 0.12 2.49 ± 0 05
23	0.86 4.31	4 3	1.53 (1.06–2.00) 13.4 (10.9–15.0)	1.14 (0.89–1.49) 2.02 (1.82–2.23)	1.33 ± 0 04 1.18 ± 0 09
28	0.84 0 0	3 4 4	1.20 (0.96–1 35) 0.16 (0.09–0.28) 0.04 (0.04–0.04)	2.13 (2.11–2.18) 1.39 (1.12–1.79) 1.83 (1.56–2.10)	1.78 ± 0 05 2.42 ± 0.17 2.30 ± 0 05

Clearance experiments.

The urine flow averaged 0.6 ml/hour (range 0.4–0.8 ml/hour). The delay time, determined as the time interval from a rapid intra-arterial injection of riboflavin until its appearance in the urine, was approximately 3 min. at this urine flow. The mean arterial blood pressure varied from 100 to 140 mmHg.

Glomerular filtration rate and clearance ratio. The gross data of all the experiments are shown in tables 1, 2 and 3. Table 1 lists the experiments with constant infusion rates, arranged in the order of increasing plasma riboflavin concentrations. Table 2 lists the experiments in which probenecid was administered, and table 3 the experiments with varying infusion rates.

Since the GFR, as determined by the inulin clearance, varied considerably during the experiments, it was perferred to indicate the riboflavin:inulin clearance ratios instead of the absolute riboflavin clearances. If no tubular transport occurred the expected clearance ratio would be 0.79.

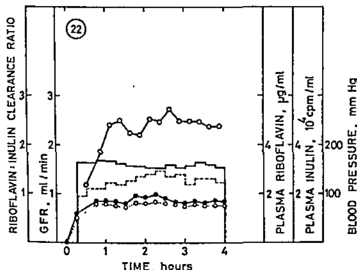


Fig. 2. Clearance experiment with a constant infusion rate of riboflavin (0.50 mg/hour). The time course of five different parameters is indicated: Riboflavin:inulin clearance ratio (columns), GFR (——○——), plasma concentrations of riboflavin (——●——) and inulin (- - - -○- - - -) and mean arterial blood pressure (broken columns).

Representative experiments with constant infusion rates and intermediate plasma concentrations are shown in fig. 2 and 3, where the time course of five different parameters is indicated. Fig. 2 illustrates an experiment with a relatively constant GFR and, consequently, with constant plasma concentrations of inulin and riboflavin. In many experiments, however, as indicated in fig. 3, the GFR varied considerably with time. It appears that the plasma concentrations of inulin and riboflavin in these experiments varied in accordance with the GFR. Experiment 19 demonstrates that the rapid variations in GFR were not associated with changes in the arterial blood pressure.

The riboflavin:inulin clearance ratio was generally much higher than 0.8 (fig. 2 and 3, table 1), ranging from 1.2 to 3.2 between individual rats. In the same animal, however, the clearance ratio was remarkably constant, even when the GFR varied considerably (fig. 3).

Effect of probenecid. Probenecid, administered intra-arterially to animals during constant rate infusions, depressed the initially high clearance ratios to values between 0.8 and 1.0 (fig. 4 and table 2). The decreased urinary excretion of riboflavin after probenecid is also reflected by the plasma riboflavin concentration, which exhibits a secondary rise. Probenecid had no effect on the GFR except after the highest dose when convulsions occurred (experiment 10). A maximum effect of probenecid was achieved 30 min. after a single injection (experiment 16) and persisted for several hours.

Relationship between clearance ratio and plasma riboflavin concentration. Because of the considerable individual variation in the clearance ratio, the relationship between the latter and the plasma concentration of riboflavin had to be examined in the same animal. Table 3 indicates the results of a series of experiments in which widely different plasma riboflavin levels were obtained by stepwise changes of the infusion rate.

In some experiments with very high plasma riboflavin concentrations (above 50 μg/ml) it was noticed that precipitation of riboflavin occurred in the urine. Post mortem analysis revealed that large amounts of riboflavin had been precipitated in the kidneys of these animals. All the results involving plasma riboflavin concentrations above 20 μg/ml have therefore been omitted.

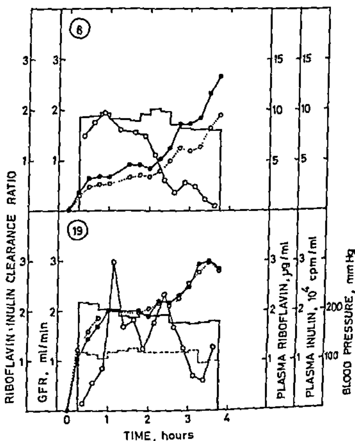


Fig. 3. Two clearance experiments with constant infusion rates of riboflavin (experiment 8, 1.05 mg/hour; experiment 19, 0.94 mg/hour). The time course of five different parameters is indicated: Riboflavin:inulin clearance ratio (*columns*), GFR (—○—) plasma concentrations of riboflavin (—●—) and inulin (----○----) and mean arterial blood pressure (*broken columns*).

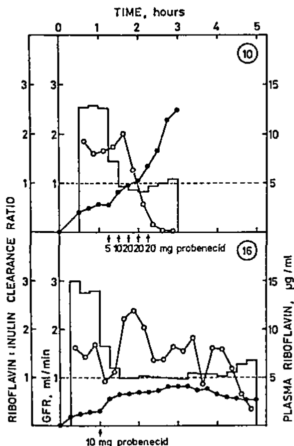


Fig. 4. Two clearance experiments with constant infusion rate of riboflavin (both 0.97 mg/hour). Probenecid was injected intra-arterially as indicated by arrows. The time course of three different parameters is indicated. Riboflavin:inulin clearance ratio (columns), GFR (——○——) and plasma concentration of riboflavin (——●——).

Fig. 5 indicates the relationship between the net tubular transport per ml of glomerular filtrate and the plasma riboflavin concentration in 8 different animals. It is evident that the tubular transport rate, at plasma concentrations where precipitation of riboflavin in the kidneys can be excluded, is increased proportionally with the plasma riboflavin in each individual rat. Thus, a tubular maximum (Tm) for the tubular secretion was not reached at the plasma concentrations available for investigation. The highest net tubular secretion rate found was 55.6 µg/min., obtained at a plasma concentration of 21.1 µg/ml and a GFR of 1.85 ml/min. (experiment 15, [illegible] ?70 g).

Three experiments carried out at very *low* plasma [illegible] less than 0.1 µg/ml (table 3, experiment 26, 27 and

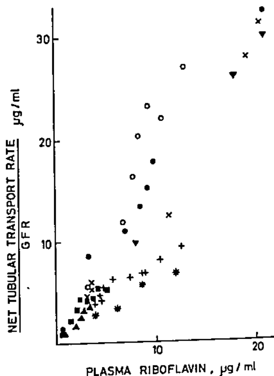


Fig. 5. The relationship between the net tubular transport per ml of glomerular filtrate and the plasma riboflavin concentration in 8 different animals.

with regard to the clearance ratio at these low levels: In experiment 26 the clearance ratio was not affected by changing the plasma riboflavin concentration. In experiment 27 the clearance ratio was significantly lower at the lowest plasma riboflavin level (0.05–0.09 µg/ml) than at the succeeding higher levels, whereas in experiment 28 the clearance ratio was greater at the low levels than at the preceeding high one. From fig. 6 it is seen that the clearance ratio, contrary to what happens at higher plasma concentrations (fig. 3), at the very low levels is affected by changes in the GFR and the urine flow. Thus, in experiment 28 strictly parallel increases are observed in the GFR, the urine flow and the clearance ratio following the injection of 1 ml of dextran-glucose.

Discussion

The finding that the riboflavin clearance in all the experiments considerably exceeds the GFR, indicates that tubular secretion is involved in the renal

excretion mechanism of this vitamin in the rat. This is supported by the action of probenecid, which decreased the riboflavin:inulin clearance ratio to 1.0 or lower. Tubular transport of riboflavin has been demonstrated in the *chicken* by RENNICK (1960) using the Sperber technique, and recently JUSKO *et al.* (1970a and b) found riboflavin clearances exceeding the GFR in the *dog* and *man*.

The relative extent of the tubular secretion varied individually as can be

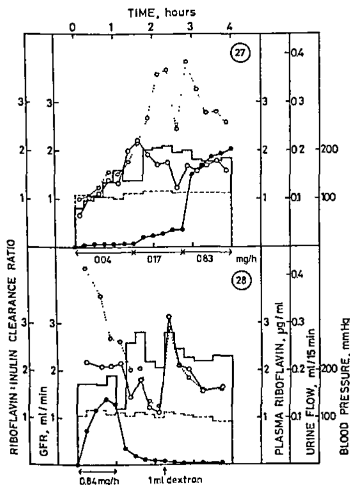


Fig 6. Clearance experiment with three different infusion rates (upper curves) and with constant infusion for 1 hour, succeeded by no infusion (lower curves). The time course of five different parameters is indicated: Riboflavin:inulin clearance ratio (columns), GFR (—○—), plasma concentration of riboflavin (—●—), *urine flow* (- - -○- - -) and mean arterial blood pressure (broken columns).

seen from the clearance ratios (1.2 to 3.2) obtained at identical plasma levels. Since these variations could not be correlated to variations of other parameters (e. g. the GFR), this probably reflects biological variation in the secretory capacity *per se*. A similar phenomenon was observed in the dog (JUSKO *et al.* 1970a).

The precipitation of riboflavin in the kidneys at high plasma concentrations made it impossible to demonstrate a possible Tm for the tubular secretion. The linearity between the tubular secretion rate (divided by the GFR) and the plasma concentration of riboflavin up to 20 µg/ml indicates that the secretory mechanism is not saturated at plasma concentrations up to this level.

The finding that the clearance ratio in individual animals remained constant during up to ten-fold variations in the GFR (fig. 3) deserves consideration. Since riboflavin is not cleared completely from the renal blood it is unlikely that changes in the renal blood flow *per se* would affect the GFR and the tubular secretion rate to exactly the same extent. A satisfactory explanation would be that the variations in GFR and the tubular secretion rate were both mediated through variations in the number of nephrons participating in the formation of urine. Evidence for such a phenomenon has been presented in the rat by LEYSSAC (1964), who demonstrated that some proximal tubules became occluded when the GFR decreased below a critical value, while others remained widely open. This critical value of the GFR, 1.2 ml/min. when converted to the animal size used by us, is within the range of the actual variations. Since the correlation between the GFR and the urine flow observed at low filtration rates (fig. 6) may also be explained on this basis, this interpretation of the results is considered to be most likely. The actual reason for the variations in the GFR was not disclosed. Very low filtration rates could occasionally be correlated to decreases in the arterial blood pressure, but as a rule there was no connection between the two parameters. It is possible that renal ischaemia, induced by surgical and anaesthesiological procedures, might have been responsible for the variations.

The fact that riboflavin is excreted as an unloaded molecule is interesting with regard to the tubular secretory mechanism involved. Since the electrical charge is thought to be essential for both cationic and anionic transport, it may be questioned whether riboflavin is transported *per se* or as an ionized metabolite. Since riboflavin-5′-phosphate is known to be accumulated in the dog renal cortex following riboflavin infusion (JUSKO *et al.* 1970a) it is not unlikely that a phosphorylation-dephosphorylation mechanism is involved in tubular secretion. The inhibitory effect of probenecid, whis has also been demonstrated in other species (RENNICK 1960; JUSKO *et al.* 1970a and b) hardly contributes to the elucidation of the secretory mechanism because of the unspecificity of this compound. The site of the secretory mechanism ap-

pears to be the proximal tubule as judged from stop-flow studies in the dog (JUSKO *et al.* 1970a).

It was not possible with the present technique to determine the riboflavin clearance at physiological plasma levels (about 10 ng/ml of free riboflavin) (STRIPP, unpublished results, 1966. The experiments carried out at the lowest plasma concentrations that could be measured (about 50 ng/ml) were inconclusive as regards the absolute value of the clearance ratio. However, at these low levels, as opposed to at the higher levels, a correlation was observed between the clearance ratio, the GFR and the urine flow. This might suggest a tubular reabsorption at the low plasma levels. Evidence for an easily saturable active reabsorption has accordingly been presented in man and dog (JUSKO & LEVY 1970). Such a reabsorption mechanism with a low plasma threshold would explain the extremely low urinary excretion of riboflavin in animals on a riboflavin-restricted diet (CHRISTENSEN 1969b). Calculations based on the plasma and urine data from a study of BURCH *et al.* (1948) have further shown that the riboflavin clearance in rats receiving limited, although sufficient, amounts of riboflavin, might even be lower than 20 ml/100 g rat/24 hours i. e. considerably less than the GFR (min. 870 ml/100 g rat/24 hours (SMITH 1951)).

The limitations of the classical clearance technique in studies of complex bi-directional transports are well known. Thus, it may be difficult to disclose a passive back diffusion of compounds which are also secreted by the tubules. This possibility might, however, be rejected in the present study with regard to the probenecid experiments (table 2). The lowest clearance ratio reached following 20 mg probenecid, was 0.82 ± 0.02 (S. E. M.), and it is reasonable to assume that this ratio was obtained during a complete blockade of the tubular transport since a much greater dose (75 mg) did not further lower the clearance ratio. The agreement between this value and the expected value, providing no tubular transport occurred (0.79), indicates that passive back diffusion of riboflavin does not occur in the tubules.

The protein binding of riboflavin in rat plasma (21 % at 37°) is similar to the binding in dog serum (19 % at 30°) which is only about half of that found in human serum (40 % at 30°) (JUSKO *et al.* 1970a; JUSKO & LEVY 1969). Since the binding is to the plasma albumins (JUSKO & LEVY 1969) and does not alter over a wide range of plasma concentration, it is likely that it occurs to the same extent at even lower plasma levels than are covered by the present binding study.

Acknowledgements

I would like to thank Miss Jette Petersen for excellent technical assistance and Hans Langgård, M. D., for fruitful discussions and critical rea- the manuscript.

REFERENCES

Ames, A. & M. Sakanoue: A new method for preparing a plasma ultrafiltrate: Distributions of 5 electrolytes. *J. Lab. Clin. Med.* 1964, **64**, 168–176.

Bray, G. A.: A simple effecient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.* 1960, **1**, 279–285.

Burch, H. E., O. A. Bessey & O. H. Lowry: Fluorometric measurements of riboflavin and its derivatives in small quanties of blood serum and cells. *J. Biol. Chem.* 1948, **175**, 457–470.

Christensen, S.: Studies on riboflavin metabolism in the rat. I. Urinary and faecal excretion after oral administration of riboflavin-5′-phosphate. *Acta pharmacol. et toxicol.* 1969a, **27**, 27–33.

Christensen, S.: Studies on riboflavin metabolism in the rat. III. Disappearance rate and urinary excretion rate of flavin after intravenous infusion of riboflavin-5′-phosphate. *Acta pharmacol. et toxicol.* 1969b, **27**, 41–48.

Jusko, W. J. & G. Levy: Plasma protein binding of riboflavin and riboflavin-5′-phosphate in man. *J. Pharm. Sci.* 1969, **58**, 58–62.

Jusko, W. J. & G. Levy: Pharmacocinetic evidence for saturable renal tubular reabsorption of riboflavin. *J. Pharm. Sci.* 1970, in press.

Jusko, W. J., B. R. Rennick & G. Levy: Renal excretion of riboflavin in the dog. *Amer. J. Physiol.* 1970a, **218**, 1046–1053.

Jusko, W. J., G. Levy, S. J. Yaffe & R. Gorodischer: Effect of probenecid on renal clearance of riboflavin in man. *J. Pharm. Sci.* 1970b, **59**, 473–477.

Leyssac, P.: The *in vivo* effect of angiotensin on the proximal tubular reabsorption of salt in rat kidneys. *Acta physiol. Scand.* 1964, **62**, 436–448.

Rennick, B. R.: Renal tubular excretion of riboflavin in the chicken. *Proc. Soc. Exp. Biol. Med.* 1960, **103**, 241–243.

Smith, H. W.: *The kidney. Structure and function in health and disease.* New York 1951.

Acta pharmacol. et toxicol. 1971, 29, 441–448.

From the Department of Pharmacology, University of Helsinki, Helsinki, Finland
(Head: M. K. Paasonen)

The Toxicity of Two MAO Inhibitors Combined with 5-HTP or L-DOPA in Anaesthetized Mice

By

P. Männistö, P. Nikki and A. Rissanen

(Received September 2, 1970)

Abstract: The combined toxicity of the MAO-inhibitors phenelzine or pargyline + 5-HTP or L-DOPA was studied in conscious and halothane or pentobarbital anaesthetized mice. The rectal temperature of the mice was followed with a thermocouple. Halothane had a transient protective effect in phenelzine (60 mg/kg) + 5-HTP (150 mg/kg) treated mice, whereas pentobarbital had no such effect. On the contrary, the toxicity was increased in pargyline (100 mg/kg) + 5-HTP (150 mg/kg)-treated mice anaestetized with halothane as compared with the toxicity in the unanaesthetized group. When L-DOPA (300 mg/kg) was given together with phenelzine (60 mg/kg) or pargyline (100 mg/kg), the toxicity was decreased in the halothane anaesthetized and somewhat less decreased in the pentobarbital anaesthetized mice as compared with the unanaesthetized mice. The results suggest that halothane decreases the toxicity of MAO-inhibitor + L-DOPA through a central action, possibly by reducing the body temperature of mice during the critical period. The temporary protection in the halothane anaesthetized phenelzine + 5-HTP group is probably due to the bronchodilation induced by halothane.

Key-words: 5-HTP – halothane – L-DOPA – MAO-inhibitors – pentobarbital.

MAO-inhibitors have been shown to cause hyperthermia in halothane anaesthesia in cats (Feldberg & Lang 1970; Summers 1969). Their possible danger and relation to the malignant hyperpyrexia in halothane anaesthesia has been pointed out (Horsey 1968; Summers 1969).

No hyperthermia was observed in mice after phenelzine + halothane, but a combined treatment with phenelzine + L-DOPA or 5-HTP caused increased shivering and hyperpyrexia following halothane anaesthesia (Nikki 1969). Since MAO-inhibitors enhance the action of anaesthetics and since the toxicity of 5-HT is increased during anaesthesia (Ta... 1968) it seemed of interest to study the combined ... of MA... HTP or L-DOPA during halothane an... temperature changes.

Material and methods

Mice weighing 15–20 g were used in groups of 20 animals. Either 60 mg/kg of phenelzine or 100 mg/kg of pargyline was injected intraperitoneally 80 min. (phenelzine) or 60 min. (pargyline) before 5-HTP 75, 100 or 150 mg/kg or L-DOPA 100, 200 or 300 mg/kg. Half of the groups received an anaesthetic, either 40 mg/kg of pentobarbital (mebumalum NFN) intraperitoneally or 2% halothane for 20 min., as seen in fig. 1. Halothane was administered with the usual anaesthesia machine (MIE Ltd.), as described previously (NIKKI 1968). All the experiments were performed at the ambient temperature of 25 ± 1 C°.

The behaviour and death of animals were followed for 4 hrs after the injection of either 5-HTP or L-DOPA. The 24-hour toxicity was also determined. The rectal temperature was measured by means of a thermocouple (Ellab, Te 3) as seen in fig. 1.

The drugs used were: L-(-)-dioxyphenylalanine (L-DOPA, F. Hoffmann-La Roche & Co., Basle), dl- hydroxytryptophan (5-HTP, Fluka AG, Buchs SG), phenelzine hydrogen sulphate (Leo AB, Hälsingborg), pargyline hydrochloride (Abbott, Brussels), pentobarbital sodium (Lääke Oy, Turku), halothane (Leiras Oy, Turku). The drugs were injected in saline (0.9%) and refer to the bases.

The statistical significance of the differencies in toxicity at 2 and 24 hrs was calculated by a modification of the t-test. Moreover the median (i. e. the time at which 50 % of animals were alive) of each group was determined.

Results

Phenelzine (60mg/kg) + 5-HTP (various doses).

Halothane anaesthesia caused a transient protection after increasing doses of 5-HTP. The protective effect was most distinct after the largest dose, 150 mg/kg of 5-HTP ($P < 0.001$) at 2 hrs (fig. 2A). Both halothane and pentobarbital caused a profound fall of rectal temperature, whereas the unanaesthetized group showed an increased temperature (to 38.7°), which fell later (fig. 2B).

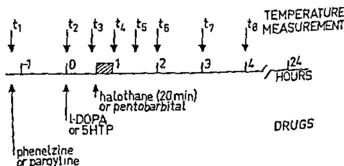


Fig. 1. The scheme of the experiment.

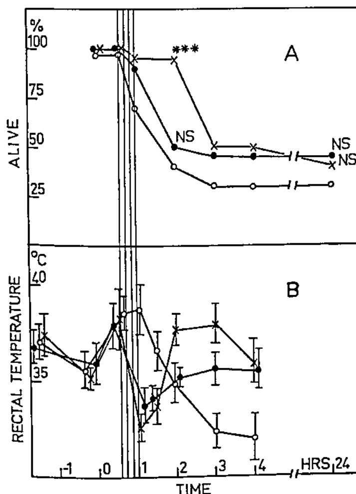


Fig 2. The mortality (A) and rectal temperature (B) of 60 mice treated with phenelzine (60 mg/kg) and 5-HTP (150 mg/kg). In 20 mice no other therapy was given (marked by O——O), 20 were anaesthetized with 2% halothane for 20 min. (X——X), and 20 were injected with 40 mg/kg of pentobarbital (●——●). The time scale is exactly the same as in fig. 1. For rectal temperature the means ± S. D. are given. The significancies of the differences in mortality at 2 and 24 hrs as compared with the control animals are shown as follows: NS: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. The period of halothane anaesthesia is indicated by four vertical lines.

Pargyline (100 mg/kg) + 5-HTP (150 mg/kg).

Pargyline-treated mice seemed to have a greater mortality after halothane than the unanaesthetized or the pentobarbital-anaesthetized mice after the same treatment (fig. 3A), but the difference was ... ne

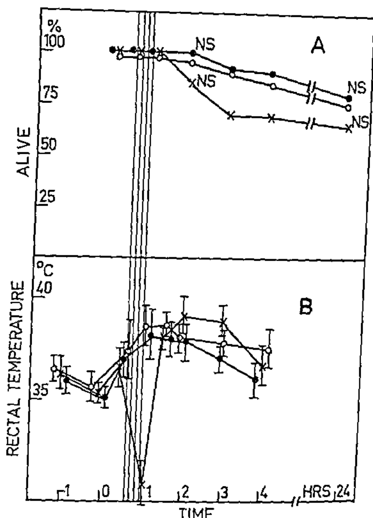


Fig. 3. The mortality (A) and rectal temperature (B) of 60 mice treated with pargyline (100 mg/kg) and 5-HTP (150 mg/kg). The three groups are otherwise the same as in fig. 2, as are also the codes.

ture curves were similar except for the transient fall due to halothane (fig. 3B).

Phenelzine (60 mg/kg) + L-DOPA (various doses).

The toxicity in the halothane-anaesthetized group did not differ from that in the unanaesthetized group when 100 or 200 mg/kg of L-DOPA was injected. After 300 mg/kg of L-DOPA both halothane ($P < 0.001$ at 2 and 24 hrs) and pentobarbital ($P < 0.001$ at 2 hrs and $P < 0.01$ at 24 hrs) had a protective effect (fig. 4A). The median for the halothane group was > 24

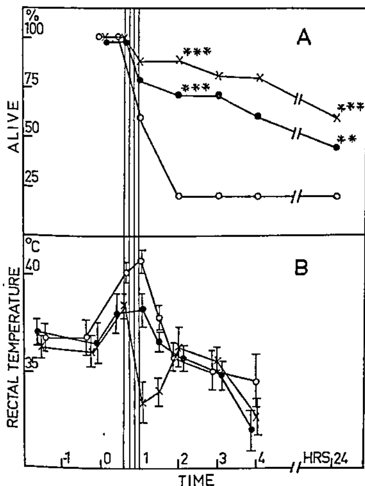


Fig. 4. The mortality (A) and rectal temperature (B) of 60 mice treated with phenelzine (60 mg/kg) and L-DOPA (300 mg/kg). The three groups are otherwise the same as in fig. 2, as are the codes.

hrs, for pentobarbital 5 hrs and for controls about 1.2 hrs. The body temperature of the halothane group fell clearly during 20 min. anaesthesia, whereas that of the pentobarbital and control group rose slightly during the same period and thereafter began to fall (fig. 4B).

Pargyline (100 mg/kg) + L-DOPA (300 mg/kg).

The same protective effect as after phenelzine was also seen after pargyline in the halothane-anaesthetized group ($P < 0.001$ at 2 and 24 hrs) (fig. 5A). The toxicity in the pentobarbital and control groups did not differ at 2 hrs, but at 24 hrs it was greater in the former group ($P < 0.05$). The median for

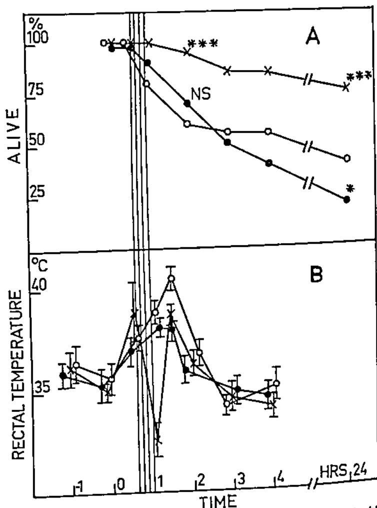


Fig. 5. The mortality (A) and rectal temperature (B) of 60 mice treated with pargyline (100 mg/kg) and L-DOPA (300 mg/kg). The three groups are otherwise the same as in fig. 2, as are also the codes.

the halothane group was > 24 hrs, for the pentobarbital group 3 hrs and for the control group 4.5 hrs. The temperature curves for the pentobarbital and control groups were similar except that the maximum temperature in the controls was 2° higher than that of the pentobarbital group 50 min. after the injection of pentobarbital (fig. 5B).

Discussion

The unanaesthetized phenelzine-mice had difficulties in breathing with a maximum at about 1 hr after the 5-HTP injection thus coinciding with the temperature maximum. The anaesthetized groups showed a fall in a temperature but only halothane and not pentobarbital, caused a temporary protection in toxicity. Halothane is known to cause a bronchodilating effect due to the beta-receptor stimulation (KLIDE & AVIADO 1967). Pentobarbital does not cause a bronchodilation. Therefore, the temporary protection in phenelzine + 5-HTP mice due to halothane is more likely to be due to bronchodilation than to a fall in temperature.

The much lower mortality in the pargyline + 5-HTP mice as compared to the corresponding phenelzine-treated mice might be due to the lack of amphetamine activity of pargyline (GOLDBERG 1964).

MAO-inhibitor + L-DOPA-treatment did not cause any breathing difficulties, but signs of central excitement and even convulsion, were sometimes seen. It is known that MAO-inhibitor + L-DOPA greatly increases the dopamine content of the mouse brain but has less effect on the noradrenaline and 5-HT content (WIEGAND & PERRY 1961). It is suggested that the protective effect of halothane and pentobarbital may be due to an inhibition of these central effects of this monoamine increase. Furthermore, the fall in temperature during anaesthesia may slow down the metabolism of these amines and thus decrease the toxicity during the critical period of the experiment. The unanaesthetized pargyline + L-DOPA mice showed less toxicity than the corresponding phenelzine + L-DOPA mice, possibly due to the lack of an amphetamine-like activity.

These results suggest, that halothane anaesthesia decreases the toxicity of combined treatment with an MAO-inhibitor + L-DOPA through a central action, possibly by reducing the temperature of mice at the critical period. The temporary protection of halothane in the phenelzine + 5-HTP-treated mice may be due to the bronchodilator action induced by halothane.

REFERENCES

Feldberg, W. & W. J. Lang: Effects of monoamine oxidase inhibitors and amphetamine on hypothermia produced by halothane. *Brit. J. Pharmacol.* 1970, 38, 181–191.

Goldberg, L. I.: Monoamine oxidase inhibitors. *J. amer. med. Ass.* 1964, 190, 456–462.

Horsey, P. J.: Hyperpyrexia during anaesthesia. *Brit. med. J.* 1968, 3, 803.

Klide, A. M., & G. M. Aviado: Mechanism for the reduction in pulmonary resistance induced by halothane. *J. Pharmacol. exp. Therap.* 1967, 158, 28–35.

Nikki, P.: Influence of some cholinomimetic and cholinolytic drugs shivering in mice. *Ann. Med. exp. Fenn.* 1968, 46, 521–530.

Nikki, P.: Biogenic amines, cold-adaption an halothane shivering in mice. *Ann. Med. exp. Fenn.* 1969, 47, 129–140.
Summers, R. J.: Effects of monoamine oxidase inhibitors on the hypothermia produced in cats by halothane. *Brit. J. Pharmacol.* 1969, 37, 410–413.
Tammisto, T.: Increased toxicity of 5-hydroxytryptamine by ethanol in rats and mice. *Ann. Med. exp. Fenn.* 1968, 46, 382–384.
Wiegand, R. G. & J. E. Perry: Effects of L-DOPA and N-methyl-N-benzyl-2-propynylamine-HCl on DOPA, dopamine, norepinephrine, epinephrine and serotonin levels in mouse brain. *Biochem. Pharmacol*, 1961, 7, 181–186.

Acta pharmacol. et toxicol. 1971, 29, 449–455.

From the Research Laboratories, AB Leo, Hälsingborg, Sweden

Effects of Long-Acting Oestrogens and a Long-Acting Corticosteroid on the Weight and Acid Phosphatase Activity of the Spleen in Male Rats

By

Maud Andersson and Jonas Müntzing

(Received September 18, 1970)

Abstract: Male rats were injected intramuscularly with 8 mg/kg polyoestradiol phosphate (PEP) (estradurin®), polydiethylstilboestrol phosphate (PSP), polyoestriol phosphate (SEP) (triodurin®) or oestradiol undecylate (EU) (progynon®-depot). After 20 days the weight of the spleen was decreased to 70 % by PEP, PSP and EU whereas SEP had no effect. The weight decrease was due to a histochemically demonstrated atrophy of the white pulp, which contained little acid phosphatase. The acid phosphatase activity per spleen was found to be unchanged although the activity per weight unit was increased. Male rats were injected intramuscularly with 100 mg/kg methylprednisolone acetate (D) (depo-medrone®). After 20 days the weight of the spleen was decreased to 60 %. The acid phosphatase activity per weight unit was not increased and the activity per spleen was decreased to 70 %. A lower dose of D (10 mg/kg) had no effect on the enzyme activity but reduced the weight of the spleen to 90 %. Adrenalectomized rats were injected intramuscularly with 10 or 1 mg/kg D. No effects on the spleen weight or phosphatase activity were found after 20 days. When PEP (8 mg/kg, intramuscularly) was injected in adrenalectomized rats treated with either 10 or 1 mg/kg D the weight of the spleen was decreased to 75 and 85 %, respectively. The acid phosphatase activity per spleen was unchanged. The results indicate that the polymerized oestrogens and EU, but not D, have a similar action on the spleen. The action of PEP in adrenalectomized rats substituted with D indicates that the effects of PEP are not mediated through the adrenal glands.

Key-words: Acid phosphatase – oestrogens – rats – spleen.

The long-acting oestrogens, polyoestradiol phosphate and polydiethylstilboestrol phosphate, which are strong phosphatase inhibitors *in vitro* (FERNÖ *et al.* 1958; DICZFALUSY *et al.* 1959), are taken up in the reticuloendothelial cells. Thus the spleen has been found to accumulate these compounds (DICZFALUSY *et al.* 1956; BENGTSSON *et al.* 1963). The uptake of polydiethylstilboestrol phosphate is mainly limited to the red pulp (BENGTSSON *et al.* 1963), which has been shown to contain large amounts of acid [illegible] activity (BLOOM & FAWCETT 1966). Since oestrogens

effects on some lymphoid tissues (DOUGHERTY 1952) it is possible that the polymerized oestrogens affect the spleen acid phosphatase due to both the enzyme-inhibiting and the oestrogenic properties of the compounds. In the present study the effects of polymerized oestrogens on the spleen weight and spleen acid phosphatase activity were investigated and compared with the effects of a long-acting oestradiol ester and a long-acting corticosteroid.

Materials and Methods

Male Wistar SPF rats, 190-210 g, were obtained from Dr. Møllegaard's breeding laboratories, Ejby, Denmark, and were kept under standard conditions during the experiment.

Polyoestradiol phosphate (estradurin®), polydiethylstilboestrol phosphate and polyoestriol phosphate (triodurin®) were synthetized at the Research Laboratories, AB Leo, Hälsingborg, Sweden. Oestrogen phosphate is slowly released in the body from the water soluble polymers, resulting in a prolonged oestrogenic effect (FERNÖ *et al.* 1958; DICZFALUSY *et al.* 1959; FREDHOLM & LINDSKOG 1969). The long-acting oestradiol ester used was oestradiol-17-undecylate (progynon®-depot, Schering AG) and the long-acting corticosteroid used was methylprednisolone acetate (depomedrone®, Upjohn). In the present paper, polyoestradiol phosphate is called PEP, polydiethylstilboestrol phosphate PSP, polyoestriol phosphate SEP, oestradiol undecylate EU, and methylprednisolone acetate D. All the injections were given intramuscularly. The injection volume was 2 ml/kg.

I. For each of the four long-acting oestrogens and for water twenty-four rats were randomly divided into 4 groups. Three of the groups were given a single injection (8 mg/kg) 20, 10 or 5 days before sacrifice. The fourth group served as control. The groups treated with the same substance and the corresponding control group were sacrificed on the same day.

II. Forty-two rats were randomly divided into 7 groups. Three of the groups were given a single injection of either water or D (10 or 100 mg/kg). The remaining groups were adrenalectomized under ether anaesthesia and given 1 % saline as drinking water throughout the experiment. At the time of operation these animals were given a single injection of either water and D (1 or 10 mg/kg) or PEP and D (8 mg/kg + 1 or 10 mg/kg). All the groups were sacrificed on the same day, 20 days after the treatment.

On the day of sacrifice the rats were decapitated and the spleen was dissected free and weighed. Furthermore, in the three non-operated groups in experiment II, the adrenal glands were removed and weighed. A small transverse cross-section of the spleen was homogenized in ice-cold distilled water in an all glass motor-driven tissue grinder. The final tissue concentration was 10 mg/ml. The homogenates were centrifuged for 15 min. at $800 \times g$ (+4°). Immediately after centrifugation the supernatants were quick-frozen and kept at −70° for two days. The time lapse between sacrifice and centrifugation was the same for all the groups.

The acid phosphatase activity in the supernatants was determined by using *p*-nitrophenyl phosphate as the substrate (Sigma Chem. Co. Tech. Bull. No. 104, 1963). The *p*-nitrophenol formed at pH 5.5 was read at 410 mμ with a Beckman DB-G Spectrophotometer. The enzyme activity is expressed as modified Sigma Units (SU) per 10 mg of wet tissue or per spleen. The significance of the differences between the values was analyzed by the use of Wilcoxon's range test (WILCOXON 1945).

For histological and histochemical studies, pieces from the spleen of rats treated 20 days before sacrifice with PEP (8 mg/kg), D (10 or 100 mg/kg) or water were fixed in Bouin, paraffin-inbedded, sectioned and stained with haematoxylin-eosin. Other pieces were fixed in cold, neutral, 10 % formalin for one day. Cryostat sections (6 μ) were made and incubated for the demonstration of acid phosphatase activity, naphtol AS-TR phosphate being used as the substrate (THOMPSON 1966).

Results

I. The median acid phosphatase activities in the untreated control groups was in the range of 9.1 to 13.3 SU/10 mg wet tissue, and the median spleen weight in the same groups was in the range of 583 to 777 mg.

The weight of the spleen decreased after treatment with PEP, PSP and EU whereas SEP had no effect (fig. 1). Although the acid phosphatase activity was increased per unit weight by the long-acting oestrogens (fig. 2), it was calculated that the activity per spleen was unchanged. Histologically, the white pulp with low acid phosphatase activity was markedly diminished 20 days after treatment with PEP.

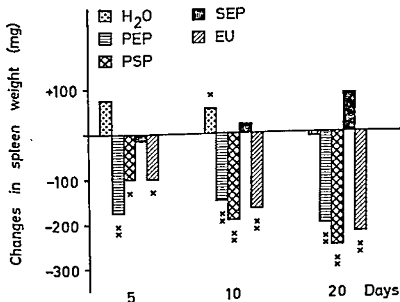


Fig. 1. Changes in spleen weight 5, 10 and 20 days after treatment with polyoestradiol phosphate (PEP), polydiethylstilboestrol phosphate (PSP), polyoestriol phosphate (SEP), oestradiol undecylate (EU) or water (x = 0.05 > P > 0.01; xx = P < 0.01).
The heights of the bars represent the difference between the median values in the treated groups and the corresponding control group.

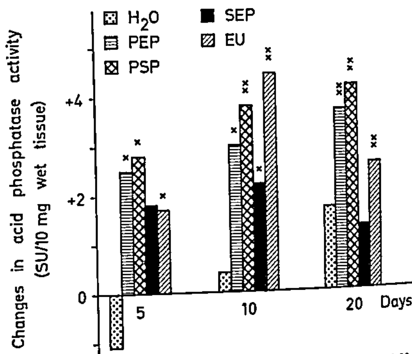


Fig. 2. Changes in spleen acid phosphatase activity per unit weight 5, 10 and 20 days after treatment with polyoestradiol phosphate (PEP), polydiethylstilboestrol phosphate (PSP), polyoestriol phosphate (SEP), oestradiol undecylate (EU) or water ($x = 0.05 > P > 0.01$; $xx = P < 0.01$).
The heights of the bars represent the difference between the median values in the treated groups and the corresponding control group.

II. The administration of 100 mg/kg of D caused the death of 2 rats. The weight of the adrenal glands and the spleen in the 4 surviving rats was markedly reduced as compared to the intact control group ($P < 0.01$) but the spleen acid phosphatase activity per weight unit was unchanged. The activity per spleen was consequently markedly reduced ($P < 0.01$). A lower dose, i. e. 10 mg/kg, of the same substance reduced the weight of the spleen ($P < 0.05$) but had no effect on the acid phosphatase activity or on the weight of the adrenal glands (table 1). Histologically, the administration of D (100 or 10 mg/kg) caused the same reduction in the white pulp as seen after PEP.

The administration of D (1 or 10 mg/kg) to adrenalectomized rats did not cause any changes in the weight of the spleen or in the phosphatase activity as compared to the intact control group. A combined treatment with D (1 mg/kg) and PEP (8 mg/kg) reduced the weight of the spleen as compared to either the intact control rats ($P < 0.05$) or the adrenalectomized rats injected with D only ($P < 0.01$). The acid phosphatase activity per weight unit was

Table 1.

Spleen weight and spleen acid phosphatase activity 20 days after treatment of intact rats with methylprednisolone acetate (D) and after treatment of adrenalectomized rats with polyoestradiol phosphate (PEP) combined with substitution doses of D.

Condition of animals	Treatment	n	Wet weight in mg Median value		Acid phosphatase activity Median value	
			Spleen	Adrenal gland	SU/10 mg wet tissue	SU/ spleen
Intact	Water	6	568	22	12.0	662
Intact	D (10 mg/kg)	6	511	23	13.0	657
Intact	D (100 mg/kg)	4	328	10	11.6	429
Adrenalectomized	Water + D (1 mg/kg)	6	644	–	12.0	733
Adrenalectomized	Water + D (10 mg/kg)	6	590	–	11.3	694
Adrenalectomized	PEP (8 mg/kg) + D (1 mg/kg)	6	482	–	14.4	697
Adrenalectomized	PEP (8 mg/kg) + D (10 mg/kg)	6	423	–	14.3	631

increased ($P < 0.05$) but the activity per spleen remained unchanged. Similar results were obtained with a higher dose, 10 mg/kg, of D combined with PEP (8 mg/kg) (table 1).

Discussion

The polymerized oestrogens used are strong phosphatase inhibitors *in vitro* (FERNÖ *et al.* 1958; DICZFALUSY *et al.* 1959; KÖNYVES 1965). Since at least two of them are accumulated in the spleen (DICZFALUSY *et al.* 1956; BENGTSSON *et al.* 1963), the acid phosphatase activity in this organ may be affected. However, with the method used in this study it was not possible to demonstrate any changes that could be attributed to the enzyme-inhibiting properties of the polymers. The effects on the spleen might instead be attributed to the oestrogenic effect of the compounds. The marked increase in the acid phosphatase activity per weight unit caused by PEP, PSP and EU was accompanied by a decrease in the weight of the spleen. As the total

activity in the spleen was unchanged it seems probable that the oestrogens do not affect the tissue containing acid phosphatase activity. This is supported by the histochemical observation that PEP caused a reduction only of the white pulp, which contained little or no acid phosphatase activity. The marked effect on the weight of the spleen was unexpected as it has been reported that although oestrogens produce an acute thymic atrophy in rats, other lymphoid structures of the body are not affected (DOUGHERTY 1952; NELSON *et al.* 1967; PEREIRA LUZ *et al.* 1969).

Although FONZO *et al.* (1967) found a decreased production of corticosteroids in female rats treated with a long-acting oestradiol ester, KITAY (1963a and b) reported an increased production of corticosteroids in male rats treated with PEP. The results of KITAY were confirmed by D'ANGELO (1968) who found that oestradiol at a certain dose level increased the plasma and adrenal corticosteroids in male rats. Thus the effects of the long-acting oestrogens on the spleen might be mediated by the adrenal glands. When a high dose of a corticosteroid, D, was administered to intact rats, the weight of the spleen was decreased in the same way as after treatment with the long-acting oestrogens except SEP. Although the histological changes were the same as those after treatment with PEP, the acid phosphatase activity per weight unit was not increased while the activity per spleen was decreased contrary to the results observed after treatment with the oestrogens.

In adrenalectomized rats a low dose of D had no effects on the weight of the spleen or acid phosphatase activity and the rats remained in good condition. Combined treatment with the same dose of D plus PEP caused atrophy of the white pulp with low acid phosphatase activity similar to the changes obtained by PEP in intact rats. This indicates that the effects on the spleen caused by PEP are not mediated by the adrenal glands.

Acknowledgement

The skilful technical assistance rendered by Miss Inger Persson is gratefully acknowledged.

REFERENCES

Bengtsson, G., S. Ullberg & T. Perklev: *Autoradiographic distribution studies after administration of a macromolecular synthetic oestrogen* (^{14}C-polydiethylstilbestrol phosphate). *Acta endocr.* (Kbh.) 1963, 43, 571–580.

Bloom, W. & D. W. Fawcett: *A textbook of histology*. W. B. Saunders Company, Philadelphia, London, 1966, 314.

D'Angelo, S. A.: *Simultaneous effects of estradiol on TSH secretion and adrenocortical function in male and female rats. Endocrinology* 1968, 82, 1035–1041.

Diczfalusy, E., U. Borell, A.-M. Magnusson & A. Westman: Distribution and probable mode of prolonged action of polyoestradiol phosphate (PEP) in spayed mice. *Acta endocr.* (Kbh.) 1956, suppl. 24, 1–52.

Diczfalusy, E., O. Fernö, H. Fex, B. Högberg & P. Kneip: High molecular weight enzyme inhibitors. IV. Polymeric phosphates of synthetic estrogens. *Acta Chem. Scand.* 1959, **13**, 1011–1018.

Dougherty, T. F.: Effect of hormones on lymphatic tissue. *Physiol. Rev.* 1952, **32**, 379–401.

Fernö, O., H. Fex, B. Högberg, T. Linderot, S. Veige & E. Diczfalusy: High molecular weight enzyme inhibitors. III. Polyestradiol phosphate (PEP), a long-acting estrogen.. *Acta Chem. Scand.* 1958, **12**, 1675–1689.

Fonzo, D., R. B. Mims & D. H. Nelson: Estrogen influence on pituitary and adrenal function in the rat. *Endocrinology* 1967, **81**, 29–33.

Fredholm, B. & M. Lindskog: Some effects of a long-acting estriol polymer, polyestriol phosphate. *Acta physiol. scand.* 1969, suppl. 330, 77.

Kitay, J. I.: Effects of estradiol on pituitary-adrenal function in male and female rats. *Endocrinology* 1963a, **72**, 947–954.

Kitay, J. I.: Pituitary-adrenal function in the rat after gonadectomy and gonadal hormone replacement. *Endocrinology* 1963b, **73**, 253–260.

Konyves, I.: En vattenlöslig estriolpolymer – polyestriolfosfat. *Tidsk. Kjemi, Bergvesen og Metallurgi* 1965, **12**, 288.

Nelson, J. H., J. E. Hall, G. Manuel-Limson, H. Freidberg & F. J. O'Brien: Effect of pregnancy on the thymolymphatic system. I. Changes in the intact rat after exogenous HCG, estrogen, and progesterone administration. *Amer. J. Obstet. Gynecol.* 1967, **98**, 895–899.

Pereira Luz, N., M. Marques, A. C. Ayub & P. R. Correa: Effects of estradiol upon the thymus and lymphoid organs of immature female rats. *Amer. J. Obstet. Gynecol.* 1969, **105**, 525–528.

Thompson, S. W.: *Selected histochemical and histopathological methods.* Thomas, Springfield 1966.

Wilcoxon, F.: Individual comparisons by ranking methods. *Biometrics Bull.* 1945, **1**, 80–82.

Acta pharmacol. et toxicol. 1971, 29, 456–470.

From the Institute of Hygiene, Karolinska Institutet and Department of Environmental Health, National Institute of Public Health, Stockholm, Sweden

Distribution of Cadmium Among Protein Fractions of Mouse Liver[1]

By

Gunnar F. Nordberg, Magnus Piscator and Birger Lind

(Received January 4, 1971)

Abstract: Liver proteins from mice injected subcutaneously with cadmium chloride (3 mg Cd/kg body weight) were separated on G-75 Sephadex at intervals 20 min.–21 days after injection. Fairly soon after the injection, cadmium was found bound to proteins of high molecular weight whereas at survival times exceeding 24 hours the cadmium was associated with a low molecular weight protein. In mice repeatedly exposed to cadmium (0.25 mg/kg, daily, 5 days/week) for periods up to 5 months, cadmium was principally bound to the low molecular weight protein, which has characteristics in common with the earlier described protein, metallothionein. The importance of "mouse metallothionein" in the toxicity of cadmium is discussed.

Key-words: Cadmium metabolism and toxicity – protein binding – metal analysis.

The distribution of cadmium in different organs and different histological organ structures has been extensively studied (Friberg & Odeblad 1957; Berlin & Ullberg 1963). From such studies it is known that cadmium accumulates in the liver and kidneys of experimental animals exposed to the metal by injection (Friberg & Odeblad 1957; Berlin & Ullberg 1963) or by the oral route (Miller *et al.* 1969). However, the studies mentioned have not revealed the distribution of cadmium in the different proteins of the organs and such data are probably of fundamental importance in understanding the toxic action of the metal. A large part of the cadmium accumulated in the livers of rabbits repeatedly injected subcutaneously with cadmium, was recovered in a protein fraction which was identified as metallothionein (Piscator 1964). On the basis of these findings it was suggested that this protein could be synthesized in the liver and act as a detoxicating agent.

---

1 Presented in part at the *General Medical Assembly*, Stockholm 1968.

It was also suggested that this protein might act as a transport protein for cadmium in the body (PISCATOR 1964). If these postulations could be proved, the protein would be of great importance in cadmium toxicology, since metallothionein has also been demonstrated in organs of normal horses (KÄGI & VALLEE 1960) and of human subjects (PULIDO *et al.* 1966). In order to gain more knowledge about the binding of experimentally injected cadmium to different protein fractions of the liver, experiments were performed involving separation of proteins from the livers of mice after single and repeated injections of cadmium chloride solution.

Material and methods

Experimental design.

Male CBA mice inbred for more than 200 generations were obtained from the Institute of Genetics, University of Stockholm. A total of 40 adult animals, weighing 24–29 grams, were used in the experiments. The mice were kept in glass cages at room temperature and were fed with ordinary mouse pellets (supplied by AB Ewos, Stockholm) and tap water. Sterile solutions of $CdCl_2$ in isotonic saline was used for all the injections.

Series I. Single injection. 18 mice were injected subcutaneously into the back with 3 mg of cadmium per kg of body weight. The mice were randomly separated into 6 equal groups for sacrifice at the following times after injection: 20 minutes, 6 hours, 24 hours, 6 days and 21 days respectively. Due to the deaths of one animal in each of the groups with the longest survival time, only two animals remained alive in each of these groups at the time of killing.

Series II. Repeated injections. *II:A:* 3 mice received daily subcutaneous injections of 0.25 mg of cadmium per kg body weight for 6 days and on the following day 3 mg cadmium per kg body weight. The mice were killed 14 days after the last injection.

II:B. 3 mice served as a control group for group II A They were kept under the same laboratory conditions and killed at the same time as the group just mentioned.

II.C. 8 mice received daily subcutaneous injections of 0.25 mg of cadmium per kg body weight, 5 days a week for a period of 5 months, i. e. a total dose of 22.5 mg per kg body weight. The mice were killed one month after termination of treatment.

II:D. 8 mice were kept under the same laboratory conditions as group II·C and served as a control for this group, they were killed at the same time as the mice in group II:C.

Preparation.

The mice were killed by prolonged ether anaesthesia. Immediately after killing, the whole liver and both kidneys were removed and the weights recorded. The kidneys and livers were frozen (−20°) and later thawed and ground together with 0.01 M TRIS buffer (pH 8.0) containing 0.05 M sodium chloride in a Potter-Elvehjelm hand homogenizer, chilled in an ice bath The homogenates were subsequently centrifuged (two or four at a time) in a refrigerated (+4°) centrifuge at 18,000 × g, for 40 minutes. Thereafter the pellet was washed by mixing with buffer and re-centrifugation for 40 minutes The supernatants from these two centrifugations were pooled and buffer added to

a total volume of 5 ml. Concentrations of cadmium in the supernatant and in the pellet were analysed by atomic absorption spectrophotometry (AAS). The remaining 4 ml of the supernatant from the livers were separated on a G-75 Sephadex column, 400 × 25 mm, by elution with 0.01 M TRIS buffer (pH 8.0) – 0.05 M-NaCl. The absorption at a wavelength of 250 nm and 280 nm was recorded in each fraction by means of a Beckman DB spectrophotometer. Thereafter the concentration of cadmium in each fraction was determined by AAS. This procedure was carried out for individual livers of group II:C, which had accumulated such considerable amounts of cadmium that analysis was easy to perform even after the dilution by gel chromatography. In series I and groups II:A and B all the livers from one group were pooled together and the analysis performed in the manner described above. In groups II:A and B the supernatant was divided into two parts, one part being run on the G-75 column with the buffer described above. The other part was separated on a G-100 Sephadex column 900 × 25 mm and eluted with a buffer of higher ionic strength, containing 0.5 M-NaCl and 0.1 M TRIS (pH 8.0). The 254 nm UV-absorption at separation on G-100 was recorded by a LKB UV-cord instrument (3 mm light path). The flow rate in the G-100 separation was 14 ml per hour. In the G-75 separation a speed of 20 ml per hour was used. To avoid any bacterial growth, 100 mg of sodium azide per litre was added to the buffer. All separations were performed at room temperature (20–24°). Organs awaiting homogenization were frozen (−20°) and stored at this temperature for up to 40 days. Supernatants awaiting separation on gel column were sometimes stored for a few days at +4°.

Metal analysis.

Cadmium and zinc were determined in whole tissue, supernatants and pellets from centrifugation by drying samples at 120–130° and subsequent ashing in a muffle furnace (Heraeus; MR 170) at 440°. The ash was then dissolved in 5 or 10 ml of 1 M nitric acid and analysed in a Perkin Elmer 303 atomic absorption spectrophotometer with recorder read-out (Perkin Elmer 303-0103 recorder read-out) and connected to a Varicord 43 (Photovolt corporation) potentiometric recorder. A Boling (three slot) burner was used under standard conditions to produce an air acetylene flame. Cadmium was analysed at the wave-length 2288 Å with slit 4 (1 mm slit opening and a bandpass of 6.5 Å), 3 times scale expansion and noise suppression 3. The minimum aspiration time was thus 15 seconds. Zinc was analysed at the wave-length 2138 Å with slit 5 (3 mm slit opening and a bandpass of 20.0 Å). Because of the high natural concentrations of zinc in biological samples, no scale expansion or noise suppression was necessary and hence the minimum aspiration time was only 1 second.

The concentrations cadmium in fractions from Sephadex separations were determined directly, without ashing. After each sample, freshly prepared standards above and below the sample value were analysed to avoid any errors from drift of the spectrophotometer. Under the above-mentioned operating conditions the detection limit of the AAS method was 0.005 p.p.m. for cadmium and 0.01 p.p.m. for zinc.

Results

General findings.

The animals in series I showed a tendency to less activity than the uninjected mice, seen already during the first 24 hours after injection. The mice

remaining alive after that time showed a progressive diminution of activity. On the 4th and 5th days a mucous secretion from the eyes was observed, sometimes so severe that the mice had obvious visual difficulties. On the 5th and 6th days when nine mice remained alive, several had these eye symptoms and some also had obvious difficulties in walking, especially because of weakness in the hind limbs. Such symptoms were also observed in two of the mice killed on the 6th day. Of the remaining 6 mice one mouse died on the 7th day and another mouse on the 10th day. These two mice were excluded from the results. After the 10th day following injection the remaining mice recovered. Approximately 14 days after the injections they had regained their original state, as far as could be judged from superficial observation and examination.

The mice in series II:A, in spite of receiving a higher total dose of cadmium, did not show any of the signs described for series I, except for a general decrease in activity on the first day after the injection of 3 mg Cd per kg body weight. However, no animals in this group died.

Animals in group II:C, like the animals in the control groups II:B and II:D showed no signs of toxicity on superficial observation and examination. All the animals in these groups survived until the day of killing.

Organ weights.

The wet weights of organs from animals of different series are seen in table 1. There was a decrease in the weights of the livers and kidneys in group I, simultaneous with the development of symptoms. 18–21 days after injection, however, a complete regeneration of the organs seems to have occurred so that their weights did not differ from those of the non-injected mice. There was also a decrease in the weight of the testicles but the results from studies on this organ will be reported in a separate paper.

Metal concentrations.

Concentrations of cadmium and zinc were analysed for pooled livers of the survival groups in series I and II:A and B, for individual mice in series II:C and D. The results are shown in table 2 and 3. Liver and kidney concentrations of cadmium show increases up to 6 days after the injection. This is clearly shown by the μg/g values, while the total amount of cadmium in the whole organ also shows a maximum at the same time. The very large rise in the concentrations of cadmium in the livers I:7–9 (24.4 μg/g) to I:10–12 (60.3 μg/g) is mainly dependent on the decrease in organ weight, since the total amount of cadmium increased only from 80 to 87 μg.

The animals from the "control" series II:B, killed at the same time as series I and series II:A, had no demonstrable amounts of cadmium either in the livers or kidneys. Animals from group II:D, not receiving any Cd-injections but which were kept for another six months showed demonstral·

Table 1.

Organ weights (gram wet weight).

Series	No.	Treatment	Survival time	Liver	Two kidneys
I	1	3 mg/kg	20 min.	1.240	0.409
	2			1.372	0.433
	3			1.119	0.494
	4	3 mg/kg	4 hrs	1.176	0.402
	5			1.298	0.365
	6			1.247	0.382
	7	3 mg/kg	24 hrs	1.180	0.381
	8			1.332	0.446
	9			0.771	0.392
	10	3 mg/kg	6 days	0.445	0.235
	11			0.499	0.261
	12			0.484	0.267
	13	3 mg/kg	18 days	1.162	0.347
	14			1.162	0.319
	15			Dead on 7th day	
	16	3 mg/kg	21 days	1.223	0.327
	17			1.252	0.346
	18			Dead of 10th day	
II A	1	6 × 0.25 + 3 mg/kg	14 days	1.035	0.273
	2			1.201	0.452
	3			0.739	0.443
II B	1	Controls		1.389	0.448
	2			1.175	0.367
	3			1.145	0.295
II C	1	0.25 mg/kg 5 days weekly, 5 months, total 22 mg/kg	Killed one month after termination of exposure	1.215	0.428
	2			1.386	0.504
	3			1.445	0.389
	4			1.389	0.550
	5			1.302	0.457
	6			1.330	0.447
	7			1.211	0.506
	8			1.153	0.436
II D	1	Controls		1.353	0.490
	2			1.383	0.524
	3			1.392	0.500
	4			1.326	0.468
	5			1.416	0.561
	6			1.551	0.476
	7			1.421	0.451
	8			1.431	0.495

Table 2.

Cadmium concentrations in organs.

		Survival time	Liver				Kidney			
			μg/g wet weight	Total μg *in organ*	μg in su-*pernatant*	% of total in *supernatant*	μg/g wet weight	Total μg in organ	μg in su-pernatant	% of total in supernatant
I	1 + 2 + 3	20 min.	11.4	42.4	18.2	42.9	3.1	4.1	1.4	34.1
	4 + 5 + 6	4 hrs	15.5	57.4	28 8	50.2	4.7	5.4	3.7	68.5
	7 + 8 + 9	24 hrs	24.4	80.2	50.0	62.3	11.0	13 5	7.5	68.2
	10 + 11 + 12	6 days	60 8	86.8	75.5	86.9	26.1	19.9	12.3	61.8
	13 + 14	18 days	21.8	50.8 (76.2)*	41.0	80.7	11.2	7.4 (11.1)*	4.5	60.8
	16 + 17	21 days	18 8	46.2 (69.3)*	31.6	68.4	7.4	5.7 (8.6)*	3.4	59.6
IIA	1–3	14 days	31.1	92.7	85 9	92.7	6.3	7.3	6.1	96.8
IIB	1–3	Control	0.0	0 0	0 0	0 0	0.0	0.0	0.0	0.0
IIC	1	*6 months' experiment*	185.5	225.4	200 0	88 7	126.7	54.2	33.4	61.6
	2		184.7	256.0	226 3	88.4	99.2	50.0	26.2	52.2
	3		166 7	240.9	214.0	89.2	122.9	47.8	38.5	80 5
	4		193 9	269.3	223.0	82.8	113.7	62.5	49.8	79.7
	5		190 8	248.5	217.0	87.5	104.2	47 6	37.8	79.4
	6		215.5	286 6	254.5	88 8	116.2	55.4	42.3	67.4
	7		236 0	285.7	200.0	69.9	110 0	55 6	37.9	68.2
	8		201.5	232.2	192.5	82.9	104.2	45 4	32.5	71.6
	Mean		196 8	255.6	215.9	84.8	112.1	52 3	37.3	70.1
IID	1	Control					0.5	0 2		
	2		0 4	0.5			1.1	0.6		
	3–8		not analyzed							

* Recalculated to three animals for comparison with animals 1–12.

Table 3.

Zinc concentrations in organs and $\frac{Cd}{Zn}$ ratios.

Series	No.	Survival time	Liver			Kidney		
			µg/g wet weight	Total µg in organ	Molar Cd/Zn ratio	µg/g wet weight	Total µg in organ	Molar Cd/Zn ratio
I	1 + 2 + 3	20 min.	22.9	85.3	0.290	18.9	25.2	0.095
	4 + 5 + 6	4 hrs	19.5	72.4	0.462	16.1	18.5	0.170
	7 + 8 + 9	24 hrs	41.5	136.1	0.342	19.4	23.7	0.330
	10 + 11 + 12	6 days	97.8	139.6	0.362	32.6	24.9	0.466
	13 + 14	18 days	36.5	84.9	0.347	36.8	24.5	0.177
	16 + 17	21 days	38.8	95.3	0.282	30.8	23.5	0.140
IIA	1–3	14 days	–	–	–	24.7	28.7	0.148
IIB	1–3	control	29.4	108.9	0.000	19.3	27.0	0.000
IIC	1	6 months' experiment	70.0	85.0	1.541	65.5	28.0	1.125
	2		170.4	236.2	0.630	67.1	33.8	0.860
	3		110.2	159.3	0.880	95.4	37.1	0.749
	4		81.6	113.3	1.382	95.1	52.3	0.695
	5		61.4	80.0	1.807	93.1	42.5	0.651
	6		75.9	101.0	1.651	–	–	–
	7		84.6	102.4	1.622	63.1	31.9	1.014
	8		52.8	60.9	2.220	–	–	–
	Mean		88.4	117.3	1.467	79.9	37.6	0.849
IID	1	Control	43.3	58.6	–	46.7	22.9	0.006
	2		69.1	95.6	0.003	–	–	–
	3–8		–	–	–	–	–	–
	Mean		56.2	77.1		46.7	22.9	

though very low concentrations of cadmium in their livers and kidneys. The animals in group II:C, which received a total of 22 mg Cd per kg body weight had high concentrations in their livers (197 µg/g) and kidneys (112 µg/g). These concentrations were considerably higher than those recorded for the animals in series I, which showed a clearly toxic reaction.

The total average amount of cadmium in the liver and kidneys from one mouse of group II:C was 308 µg, which is about half of the total amount injected during 6 months (590 µg per mouse). Of a total amount of cadmium (220 µg) given as a single dose to the three animals No. 10, 11 and 12 of series I, 107 µg was recovered in the livers and kidneys. In this series too about half of the injected dose was retained in the liver and kidneys.

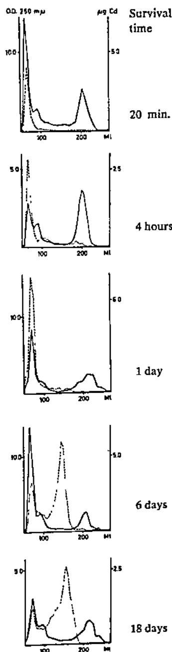


Fig. 1. G-75 Sephadex separation of liver proteins from mice of series I which received a single subcutaneous injection of $CdCl_2$ (3 mg Cd/kg body weight) and were killed at different intervals after injection.

------ μg Cd in 5 ml fraction (determined by AAS.)

——— UV-absorption (O.D.) at 250 nm.

Concentrations of zinc in organs and the molar Cd/Zn ratio are given in table 3. With increasing doses of cadmium, increasing Cd/Zn ratios are observed.

Separation of organ proteins by centrifugation and gel filtration.

Amounts of cadmium in supernatant after centrifugation at 18,000 × g are given in table 2. The amount found in the pellet is the difference between total µg in the organ and µg in the supernatant and has therefore not been put into the table as a separate column. The percentage of total cadmium found in the supernatant is 50 % or lower for the short survival times after injection, but at 24 hours and later, the main part of the total cadmium in the organs is found in the supernatant. In the longest survival times of series I and series II:A and C, more than ⅔ of the cadmium is found in the supernatant of the livers.

The results of gel filtration are seen in fig. 1 where diagrams on the cadmium content in fractions and UV-250 nm absorption curves are shown for the different survival times of series I. The diagram could not be completed because a technical accident caused the loss of a number of fractions from 21 days' animals of this series. In fig. 1 no clear peak in the 250 nm curve corresponded to the low m. w. Cd peak. However, in the 6 days' diagram a small peak was present, though it is not evident in fig. 1. Simultaneously with the recording of 250 nm absorption an 280 nm absorption was also recorded. The small peak in the 250 nm curve of the 6 days diagram was completely absent in the 280 nm curve. The recovery of cadmium in the series of fractions obtained after separation on gel column was always between 80–120 per cent of the input.

As mentioned under section "Methods" the supernatant from pooled livers of series II:A was divided into two parts. One part was separated on G-100 Sephadex with buffer of higher ionic strength. A corresponding part of the supernatant from livers of series II:B was separated on the same G-100 column. The diagrams from these gel filtrations are seen in fig. 2. The 254 nm curves are identical except for a peak which appears at an elution volume of 360–380 ml in the curve from animals of series II:A. With the G-100 system used, this elution volume indicates that the protein has a low molecular weight.

The fractions corresponding to the respective peaks in fig. 2 A were pooled and analysed for cadmium. From these analyses, it was seen that the cadmium concentration did not differ from the artificial reading caused by light scattering, except for the low molecular weight peak, which contained a total of 5 µg Cd. However, the quantitative analysis of the cadmium content of different fractions from this separation was difficult because of the light scattering from the strong sodium chloride concentration in the buffer. The cadmium

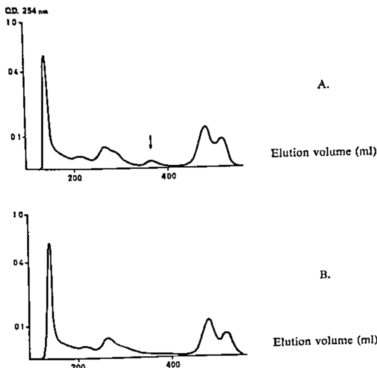


Fig. 2A. Sephadex G-100 separation of liver proteins from mice of group IIA receiving subcutaneous injections i. e. 6 times 0.25 mg Cd/kg body weight and thereafter a single dose of 3 mg Cd/kg body weight. The mice were killed 14 days after the last injection. ↓ indicates low molecular weight protein with high cadmium content.
Fig 2B. Sephadex G-100 separation of liver proteins from unexposed CBA mice (group IIB)

concentrations in different fractions are therefore better illustrated in the diagram of fig. 3, describing the separation on G-75 and with elution medium of a lower sodium chloride concentration. In this diagram the 250 and 280 nm absorption is illustrated for different protein fractions. It is seen that the low molecular weight peak displays a higher absorption for ultraviolet light of 250 nm than for such light of 280 nm. Gel filtration studies were also performed on the single livers from the animals in series II:C. Essentially they displayed the same pattern as illustrated in fig. 3. It was therefore considered unnecessary to present all these curves.

Further characterization studies.

In order to characterize further the low molecular weight cadmium-binding protein found in the livers of cadmium-exposed mice, the cadmium-contain-

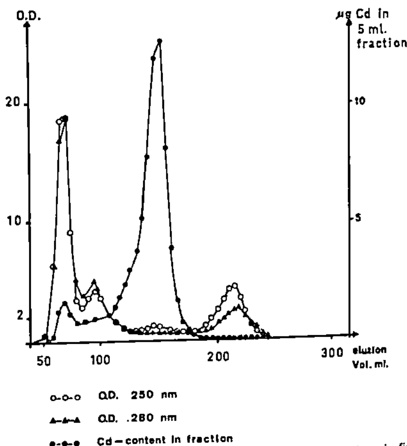


Fig. 3. Separation of supernatant from the livers of the same animals as in fig. 2A Separation on G-75 Sephadex.

ing fractions corresponding to the low molecular weight peak were pooled from groups II:A and C and a small part of the pool concentrated on a collodion membrane (Sartorius' membranfilter GmbH, Göttingen, West Germany). The concentrate was thereafter subjected to paper electrophoresis. A band appeared clearly in the β-region. The main part of the pool was concentrated on a Diaflo ultrafiltration cell with UM-2 filter and subjected to a preliminary study by the isoelectric focusing method of VESTERBERG (1968).[1] The results of these studies will be reported in detail later when data from other series of mice have been completed. In the first fractionation, however,

[1] Separation performed by Dr. O. Vesterberg.

a pI of 5.7 was obtained and a UV-absorption $\frac{250\ \text{nm}}{280\ \text{nm}}$ ratio of 5 for the protein from groups A and C of series II.

Discussion

As outlined in the introduction, the aim of the present investigation was to take a first step in a programme to correlate toxic manifestations of cadmium to the accumulation and binding of the metal in the organs. From the description of the toxic manifestations in the form of symptoms, signs and decrease in organ weights, it is clear that the dose given to series I was toxic. Out of six mice expected to survive for more than 6 days, two died.

Animals of series II:A, which received pretreatment with 6 smaller doses of cadmium and thereafter received the same large dose as series I (3 mg/kg body weight), did not die and did not develop symptoms in spite of having received a higher total dose than the animals which received only one dose. These data suggest a protective action of pretreatment against the toxicity of a single large dose of the metal and are in concordance with earlier observations by GABBIANI *et al.* (1967). They showed that pretreatment with cadmium induced resistance to the lethal action of a second dose of cadmium.

The liver and kidney are the organs which accumulate the highest cadmium concentrations in the body (e. g. FRIBERG & ODEBLAD 1957; BERLIN & ULLBERG 1963). In the present study about 50 per cent of the injected amount of cadmium was recovered in the liver and kidneys of the animals from both series. This fact shows that cadmium is very efficiently retained in the mice. It also shows that the total dose of the metal retained is not correlated to the appearance of toxic manifestations. In the case of single exposure, signs of toxicity were apparent with a total of 35 μg Cd per mouse in the liver and kidneys, whereas no similar manifestations were seen in animals from group II:C, with 250 μg Cd per mouse in the liver and kidneys. It is difficult to account for this variance in tolerance, but a few observations made in the present study could possibly be related to the phenomenon. In the case of repeated exposure a very large part of the cadmium was found in the supernatant. Most of this cadmium was bound to a low molecular weight protein. The formation of this low molecular weight cadmium-protein complex is not instantaneous, but occurs some time (fig. 1) after the injection. If the low molecular weight cadmium-protein complex is less toxic than other forms of cadmium – a theory which was previously advanced (PISCATOR 1964) and recently supported by enzyme studies (WISNIEWSKA-KNYPL & JABLONSKA 1970) – the finding of a large part of the cadmium in this ·
at often repeated exposure may constitute an explanation for the di

in tolerance. Pretreatment might stimulate the synthesis of the cadmium binding protein in the liver so that a large dose is more easily taken care of. However, further studies are necessary before these relationships can be definitely proved.

In the following some observations related to the appearance and nature of the low molecular weight cadmium-protein complex will be discussed.

A very prominent peak is seen in the Cd-curve (fig. 1) at survival times exceeding 24 hours, but no corresponding peak in the 250 nm curve was observed in the gel filtration diagrams. A very small peak seen in the 250 nm curve of the 6th day diagram, did not occur at 280 nm. If cadmium is bound to metallothionein – a protein which has previously been shown to have the capacity of binding at least 5 per cent of its weight as cadmium (KÄGI & VALLEE 1960 and 1961) – the amounts of this protein necessary for binding the amounts of cadmium present in low molecular weight fractions of series I are so small that they would be hard to detect in the 250 nm curve. The amount of Cd-protein complex formed in response to a single dose of 3 mg Cd/kg body weight was evidently on the border of what could be detected by UV-absorption.

According to the theory advanced by one of us (PISCATOR 1964) cadmium should stimulate the formation of metallothionein in the liver. Though this theory has never been proved, we tried to produce more of the cadmium-binding protein in mouse liver by exposing mice repeatedly. The results showed that this was the case. The data from series II refers to such exposure. A specific binding of cadmium to fractions corresponding to low molecular weight is seen in fig. 3, and the amount of cadmium as well as the amount of protein (clear peak in 250 nm curve) in the low molecular weight fraction was higher than in the fractionation obtained from a single exposure.

In an attempt to demonstrate the difference between cadmium-injected mice and unexposed mice, the 253 nm absorption curves from G-100 separation of liver proteins from series II:A and II:B respectively were compared. The curves are nearly identical except for a cadmium containing peak at elution volume 390 ml. In fig. 3 the difference in the 250 nm and 280 nm absorption for this low molecular weight peak is evident.

The appearance of the low molecular weight Cd-protein complex appearing as a peak not seen in unexposed mice could possibly reflect an increased amount of Cd-metallothionein complex, formed as a result of cadmium injection. The formation of the complex could be due to the synthesis of metallothionein in the liver. However, there are alternative explanations. Because metallothionein has no absorption at 254 nm, except when it is bound to cadmium, the peak could be the result of the binding of cadmium to metallothionein. Such an explanation presumes the presence of considerable amounts of non-cadmium containing forms of metallothionein in the

liver prior to Cd-exposure. The fact however, that the binding of cadmium to the low molecular weight fraction did not appear during shorter survival times in the animals subject to a large single dose of cadmium only, is evidence against the possibility that low molecular weight metallothionein is present in considerable amounts in the livers of non-cadmium-exposed mice. After this investigation was completed, other investigators have presented evidence supporting the theory of a synthesis of metallothionein in response to cadmium exposure (SHAIKH & LUCIS 1970).

The fact that the protein which bound cadmium at survival times exceeding one day appeared at an elution volume indicating a low molecular weight and moreover presented a higher absorption at 250 nm than at 280 nm, indicates that it is similar to metallothionein, i. e. a low molecular weight protein which was originally purified from equine kidney by KÄGI & VALLEE (1960 and 1961) who also characterized the protein in detail. One of its characteristics *is that it does not contain any aromatic amino acids and* therefore lacks absorption at 280 nm.

The data from paper electrophoresis showing that the low molecular weight protein from the mice migrated as a β-protein and the data from purification of the protein by isoelectric focusing showing a 250/280 nm ratio of 5, are characteristics which suggest a similarity between the cadmium-binding low molecular weight mouse liver protein and the previously described metallothionein. The isoelectric point 5.7 is somewhat different from the pI (4.5–5.5) reported by KÄGI & VALLEE (1960 and 1961) for metallothionein obtained from equine kidney. In addition to the difference in species, the method of isoelectric focusing used in this study may have contributed to the different pI. Even if the mouse liver protein is not yet well characterized, it seems to have many similarities to metallothionein, and we should therefore like to suggest the name "mouse liver metallothionein" for this protein. Further characterization of cadmium binding proteins from different species is being investigated in our laboratory. The results of these investigations may answer some of the questions raised in connection with the discussion on the results from the presently reported data.

Acknowledgements

Grants from the Swedish Medical Research Council Project B67-13X-775-02 and B68-13X-775-03 and the Folksam Insurance Company are gratefully acknowledged.

REFERENCES

Berlin, M. & S. Ullberg: The fate of Cd-109 in the mouse. An autoradiographic study after single intravenous injection of Cd-109 Cl_2. *Arch. Environ. Health* 1963, **7**, 686-693.

Friberg, L. & E. Odeblad: Localization of Cd-115 in different organs. *Acta Pathol. et Microbiol. Scand.* 1957, 41, 96–98.

Gabbiani, G., D. Baic & C. Déziel: Studies on tolerance and ionic antagonism for cadmium or mercury. *Canadian Journ. Physiol. Pharmacol.* 1967, 45, 443–450.

Kägi, J. H. R. & B. L. Vallee: Metallothionein: A cadmium and zinc containing protein from equine renal cortex. *J. Biol. Chem.* 1960, 235, 3460–3465.

Kägi, J. H. R. & B. L. Vallee: Metallothionein: A cadmium and zinc containing protein from equine renal cortex. II. Physicochemical properties. *J. Biol. Chem.* 1961, 236, 2435–2442.

Miller, W. J., D. M. Blackmon, R. P. Gentry & F. M. Pate: Effect of dietary cadmium on tissue distribution of ^{109}cadmium following a single oral dose in young goats *Journal of Diary Science* 1969, 52, 2029–2035.

Piscator, M.: On cadmium in normal human kidneys together with a report on the isolation of metallothionein from livers of cadmium exposed rabbits. *Nordisk Hygienisk Tidskrift* 1964, 45, 76–82.

Pulido, P., J. H. R. Kägi & B. L. Vallee: Isolation and some properties of human metallothionein. *Biochemistry* 1966, 5, 1768–1777.

Shaikh, Z. A. & O. J. Lucis: Induction of cadmium binding protein. *Fed. Proc.* 1970, 29, 301.

Vesterberg, O.: Isoelectric focusing of proteins. *Svensk Kem. Tidskrift* 1968, 80, 213–226.

Wisniewska-Knypl & J. Jablonska: Selective binding of cadmium *in vivo* on metallothionein in rat's liver. *Bull. Acad. Pol. Sci.* 1970, 18, 321–327.

Acta pharmacol. et toxicol. 1971, 29, 471–480.

From the National Institute of Occupational Health,
Stockholm, Sweden

Metabolism of 1,1,1,2-Tetrachloroethane in the Mouse

By

Sven Yllner

(Received July 28, 1970)

Abstract: 1,1,1,2-tetrachloroethane was given subcutaneously to mice (1.2–2.0 g/kg) and the excretion was followed for 3 days. About half of the dose (21–62 %) was expired unchanged. The part metabolized was excreted mainly as trichloroethanol (17–49 % of the dose), and to a lesser extent as trichloroacetic acid (1–7 %). The metabolites were identified by paper chromatography, gas chromatography and mass spectrometry and the Fujiwara reaction was used for the quantitative determination of trichloroacetic acid and of trichloroethanol after oxidation to this acid. It is suggested that the initial metabolic reaction is a hydrolytic fission of a carbon-chlorine bond with the formation of trichloroethanol. Trichloroethanol was the precursor of trichloroacetic acid.

Key-words: Tetrachloroethane – metabolism.

As technical solvents the aliphatic chlorohydrocarbons have many important industrial applications, but the uses of several of these substances are greatly restricted by their toxicity. The chlorinated aliphatic hydrocarbons usually possess a powerful narcotic effect and in addition show a varying toxicity with regard to e. g. injuries to the parenchymatous organs. In contrast to the extensive toxicological research on these compounds their fundamental biochemical reactions have received scant attention.

It was found by Heppel & Porterfield (1948) that when incubated with slices of rat liver and kidney a number of aliphatic halogen compounds were dehalogenated. A preparation of liver-cell supernatant had the same properties as slices, and when bromochloromethane was used as substrate, formaldehyde was formed. Dibromo- and dichloromethane reacted similarly. Other compounds found to be dehalogenated were ethyl bromide, 1-
ethane, 1,2-dichloroethane, 1-bromo-2-chloroethane and ch
ever, no organic reaction products of these compounds were

It is evident from these results that certain tissues contain an enzyme system capable of catalyzing the hydrolysis of the carbon-halogen bond in alkyl-halide compounds. This also seems to be an important reaction in the metabolism of 1,1,1,2-tetrachloroethane. The principle results have been reported in a preliminary paper (YLLNER 1963).

Materials and Methods

Boiling points are uncorrected and measured at atmospheric pressure. The elementary analysis was made by Alfred Bernhardt, Elbach über Engelskirchen, West-Germany.

1,1,1,2-Tetrachloroethane was prepared as described by PRINS (1922) by adding hydrogen chloride to trichloroethylene in the presence of anhydrous aluminium chloride. The crude product was purified by fractional distillation, and the purity of the final product (b. p. 130°) was checked by gas chromatography. Less than 0.1 per cent of impurities and 0.03 per cent of trichloroethylene were found.

Trichloroacetic acid methyl ester. Trichloroacetic acid was dissolved in a small volume of methanol and an etheral solution of diazomethane was added in slight excess. The reaction mixture was evaporated *in vacuo* (10 mmHg) at room temperature, and the remaining ester purified by fractional distillation. B. p. 154–6°.

2,2,2-Trichloroethanol. A commercial product (Fluka AG) was purified by filtration through charcoal and then by distillation *in vacuo.* B. p. 152–3°. Gas chromatography showed that the trichloroethanol still contained about 2 per cent of impurities. The product was not submitted to further purification.

$C_2H_3OCl_3$: Calculated: Cl 71.2 per cent. Found: Cl 69.9 per cent.

Other chemicals used were trichloroethylene (analytical grade, Baker Chemicals), chloroacetic acid (analytical grade, Merck AG), dichloroacetic acid (technical grade), and trichloroacetic (analytical grade, Merck AG).

Animals. Female albino mice of the NMRI strain (Naval Medical Research Institute, Bethseda, USA) weighing 20–30 g were used. They were fed *ad libitum* on water and a standard diet. (KYLIN *et al.* 1963).

Gas chromatography. Gas chromatography was performed with:

(A) A Perkin-Elmer fractometer 116 equipped with a thermistor detector and a 5 mV recorder. With this chromatograph, 2 m columns of aluminium tubes (¼" inner diameter) containing the following stationary phases were used: (1) Paraffin, m. p. 50–52°; (2) polyethylene glycol 1500; (3) octylphthalate; (4) silicone SE 52. The stationary phases were absorbed on 10 times their weight of Chromosorb W, 60–80 mesh. Carrier gas:helium.

(B) An Aerograph Hy-Fi, model 600, equipped with an electron-capture detector (Wilkins Instrument and Research Inc., 02–104) and a 1 mV recorder. Columns (1.5 m) of ⅛" stainless steel tubes containing the following stationary phases were used: (5) methyl silicone SF 96; (6) polyethylene glycol 1500. The stationary phases were absorbed on ten times their weight of Chromosorb W, 100–120 mesh. Carrier gas: nitrogen.

Mass spectrometry. Metabolites were identified by means of combined gas chromatography and mass spectrometry with the LKB instrument No. 9000. Carrier gas: helium.

The metabolites to be identified were introduced as 0.1–0.5 per cent solutions in ether. Identities were established by comparison with mass spectra obtained from authentic samples by the same procedure. The gas chromatographic separation of 1,1,1,2-tetrachloroethane was carried out on a column containing 1 per cent silicone SE 30 on

Chromosorb W at 80°. Trichloroethanol and trichloroacetic acid methyl ester were separated on a column containing 10 per cent polyethylene glycol 1500 on Chromosorb W, the alcohol at 145° and the ester at 70°.

1,1,1,2-Tetrachloroethane in neutral aqueous solution. 1,1,1,2-Tetrachloroethane (1 ml) was dissolved in 100 ml of a 0.2 M phosphate buffer (pH 7.0) containing 50 per cent (v/v) of ethanol. The solution was incubated at 37° and 4-ml samples were drawn for the determination of chloride ions by the Volhard titration.

The solution was also examined for tetrachloroethane Four milliliter specimens were added to 4 ml of hexane and 100 ml of water and the mixtures were shaken. The tetrachloroethane contents of the hexane layers were determined by gas chromatography with column no. 3.

1,1,1,2-Tetrachloroethane and cysteine. Sodium hydroxide was added to a solution of 1,1,1,2-tetrachloroethane and L-cysteine (equimolar amounts) in water-ethanol and in a nitrogen atmosphere. Samples were analyzed for cysteine by iodometric titration.

Dosage and collection of metabolites. 1,1,1,2-Tetrachloroethane was administered by subcutaneous injection in the scapula region with a calibrated 50 µl Hamilton syringe. After dosing, the mouse was placed in an all-glass chamber from which the urine, faeces and volatile metabolites were collected simultaneously. Each experiment was run for 3 days, during which time the mouse received 5 per cent glucose solution *ad libitum* but no food. The small amounts of faeces were homogenized with the urine and analysis of the excreta was made daily

The glass chamber was supplied with a constant slow current of dry air free of carbon dioxide. To collect volatile compounds in the air leaving the chamber the following 3 trapping devices were used:

(1) The air was passed through an ice-cooled glass thimble trap containing cellosolve (10 ml) and then through a similar trap containing iso-octane (15 ml) kept at − 80°. By means of these traps trichloroethylene could be quantitatively retained. The contents of the traps were mixed and added to 500 ml of water and the mixture was shaken vigorously. The iso-octane layer was then examined for chlorinated compounds by gas chromatography with the electron-capture detector (columns 5 and 6).

(2) For the mass spectrometric identification exhaled tetrachloroethane was trapped by passing the air through a glass U-tube (4 mm inner diameter) containing a 10 cm filling of 10 per cent silicone SE 30 on Chromosorb W, and kept at − 80°. The absorbed tetrachloroethane was transferred to ice-cooled ether by means of a stream of air passed through the U-tube heated to 100° 1,1,1,2-Tetrachloroethane in the ether solution was identified by mass spectrometry as described above.

(3) To collect 1,1,1,2-tetrachloroethane for quantitative determinations the air was first passed through concentrated sulphuric acid and then through a layer of magnesium perchlorate (Drierite) to remove traces of humidity. The dried air was finally passed through a small sintered glass thimble trap containing 0 6 ml of cumene cooled with dry ice in alcohol. The efficiency of this trap was checked by evaporating known amounts of tetrachloroethane in the empty glass chamber under identical conditions. The cumene was found to contain 93–98 per cent of the tetrachloroethane by gas chromatography.

Determination of expired 1,1,1,2-tetrachloroethane. Samples of the cumene solutions (see above) were analyzed by gas chromatography using column no. 1 at 150°. The samples (20 µl) were introduced into the chromatograph with a Hamilton syringe (50 µl) equipped with a Chaney adapter and the peak height was measured at a suitable

A standard solution of 1,1,1,2-*tetrachloroethane* in cumene was used as a reference for each sample. Three determinations were made with each cumene solution. Within the concentration interval used the ratio between peak height and tetrachloroethane concentration was found to be constant within the error of determination. Tests of the reproducibility in series of identical samples showed that the range of differences in peak height from the mean value was within ± 2 per cent of the value.

Analytical procedures for urinary metabolites.

1. *Paper chromatography.* Urine from a mouse dosed with 20 µl of 1,1,1,2-tetrachloroethane was collected for 48 hours. The urine was passed through a cation-exchange resin (Dowex 50 W – X 4) in the H^+ state, and then evaporated to dryness *in vacuo* to remove hydrogen chloride. The residue was dissolved in a small volume of water, and examined by paper chromatography. Paper chromatograms were prepared on Whatman No. 1 filter paper using the descending technique and the following solvent systems: (A) Butan-1-ol : 1.5 N ammonium hydroxide (1 : 1 v/v). (B) Propan-1-ol : concentrated ammonium hydroxide (7 : 3 v/v). (C) Acetic acid : butan-1-ol : water (1 : 4 : 5 v/v). Similarly treated urine from a control animal was used as reference. The chromatograms were dried in air and developed with ninhydrine (0.2 per cent in ethanol containing 1 per cent pyridine), with the $K_2Cr_2O_7$-$AgNO_3$ reagent of KNIGHT & YOUNG (1958), or with the silver nitrate-ammonium hydroxide reagent of MAYER (1957).

To bring about hydrolysis of conjugates, treatment with hydrochloric acid (final strength 3 N) for one hour at 100° in sealed ampoules was performed with urine or extracts from paper chromatograms.

2. *Isolation and identification.* The urine from 40 mice each receiving 30 µl of 1,1,1,2-tetrachloroethane by subcutaneous injection was collected for 48 hours. The urine was acidified with sulphuric acid and extracted continuously with ether overnight. The ether solution was then extracted with 0.15 N sulphuric acid and the ether-soluble acids were extracted from the sulphuric acid solution by the rocking-vessel procedure described below. The alkaline extract was passed through a cation-exchange resin (Dowex 50 W – X 4) in the H^+ state, and the solution of free acids evaporated to dryness *in vacuo*. The residue was treated with methanol and filtered, and a slight excess of diazomethane in ether was added to the methanolic solution. The reaction mixture was evaporated to a syrup at room temperature *in vacuo*, dissolved in ether (2 ml), and analyzed for trichloroacetic acid methyl ester by gas chromatography (column 3) and mass spectrometry.

Trichloroethanol was isolated from urine after hydrolysis with β-glucuronidase as described by DANIEL (1963). Urine obtained as described above was incubated at 37° for 48 hours with an equal volume of 0.2 M acetate buffer (pH 4.5) containing a β-glucuronidase preparation (25,000 Fishman units; Nutritional Biochemical Corp.). The trichloroethanol liberated was separated from the reaction mixture by steam distillation and the distillate extracted 3 times with ether. The combined extracts were evaporated to 10 ml, and examined for trichloroethanol by gas chromatography (column 4) and mass spectrometry.

3. *Determination of trichloroacetic acid.* Trichloroacetic acid was extracted quantitatively from urine by means of a rocking, two-container vessel as described by WIDMARK (1932). To one of the containers urine (3 ml) and 0.6 N sulphuric acid (1 ml) were added, and to the other, 0.1 N sodium hydroxide (2.5 ml); 50 ml of ether was added as communicating solvent, and the vessel was rocked for 16 hours, 12 rockings

per min.* Trichloroacetic acid in the alkaline recipient was then determined by the Fujiwara reaction as described by SETO & SCHULTZE (1956b). The absorbance was measured at 530 mμ with a Beckman DU spectrophotometer.

4. *Determination of trichloroethanol.* 2,2,2-Trichloroethanol was determined by the method of SETO & SCHULTZE (1956b). By oxidation of the urine with a chromic oxide – nitric acid mixture, the trichloroethanol and its conjugate were converted to trichloroacetic acid. The amount of total trichloroethanol was calculated from the difference in trichloroacetic acid after and before oxidation. The effect of the oxidation mixture on the trichloroacetic acid in this procedure resulted in some loss, and the yield was 85 per cent. However, for the oxidation of trichloroethanol the yield of trichloroacetic acid was only 77 per cent. Correction for this difference was therefore made in the determinations.

Determination of urinary trichloroacetic acid from mice dosed with trichloroethanol. Eight mice were given 2,2,2-trichloroethanol (5 μl, 52 μmoles) by subcutaneous injection. All the animals were deeply anaesthetized and 3 of them died within 2 days. From each of the survivors urine was collected for 48 hours. The trichloroacetic acid in the urine was assayed as described above.

Results

In vitro experiments. No reaction was observed when 1,1,1,2-tetrachloroethane was dissolved in a phosphate buffer solution of pH 7 at 37°. The concentration of the tetrachloroethane remained unchanged, and there was no formation of chloride ions during 48 hours. Furthermore, the tetrachloroethane did not react with cysteine even in strongly alkaline solution.

Volatile metabolites. Gas chromatographic examination of volatile metabolites isolated from the expired air from a mouse receiving 20 μl of 1,1,1,2-tetrachloroethane showed a small peak corresponding to trichloroethylene and equivalent to less than 0.02 per cent of the tetrachloroethane dose. Otherwise 1,1,1,2-tetrachloroethane was the only expired compound found in the traps. The identity of excreted 1,1,1,2-tetrachloroethane was confirmed by mass spectrometry.

Urinary metabolites. Paper chromatograms (solvent systems A and C) were prepared with urine from a mouse treated with tetrachloroethane. Spraying with ninhydine or the $K_2Cr_2O_7$–$AgNO_3$ reagent did not disclose any difference from chromatograms similarly prepared with urine from control animals. Treatment of urine with hydrochloric acid at 100° before the chromatographic examination gave no additional information with these reagents. There were apparently no mercapturic acids or S-substituted derivatives of cysteine, but the formation of such metabolites cannot be excluded, since they could be retained by the ion-exchange resin. S-carboxy methylcysteine is not retained, however.

* This extraction method has been used for many years at the National Institute of Occupational Stockholm, for routine determinations of trichloroacetic acid in urine. The extraction is cause certain compounds in urine have a serious effect on the Fujiwara reaction

When the $AgNO_3$ – ammonium hydroxide reagent was used two spots were obtained that were not found in chromatograms of urine from the control animals. The spot with the higher R_f values (0.50 and 0.81 in solvent systems A and B, respectively) was indistinguishable from trichloroacetic acid.

The material due to the other spot (R_f 0.18 and 0.66 in solvents A and B, respectively) was eluted with water from the appropriate zone and heated in 3 N hydrochloric acid at 100° for one hour in a sealed ampoule. Extraction with ether and examination by gas chromatography (electron-capture detector, columns nos. 5 and 6) showed a peak corresponding to 2,2,2-trichloroethanol.

Trichloroacetic acid was extracted from the urine and methylated with diazomethane. Gas chromatography showed a yield of 15 mg of trichloroacetic acid methyl ester from a total of 1.2 g of tetrachloroethane administered to 40 mice. Trichloroethanol was extracted from similarly obtained urine treated with β-glucuronidase. Examination by gas chromatography showed a yield of 150 mg. The identities of trichloroacetic acid methyl ester and trichloroethanol were established by mass spectrometry.

Quantitative determinations of metabolites. When 1,1,1,2-tetrachloroethane was given parenterally to mice part of it was excreted unchanged, probably in the breath, and the metabolites 2,2,2-trichloroethanol and trichloroacetic acid were found in the urine. The results of the quantitative determinations of these metabolites, and of tetrachloroethane excreted unchanged are shown in table 1. During the first 3 days the average total yield was 78 per cent (range 72–82). The fraction of the dose excreted unchanged was considerable, on average 48 per cent (range 21–62), and small amounts of tetrachloroethane were still being excreted 3 days after administration of the substance.

The expired air possibly contained a trace of trichloroethylene, but it did not exceed the amount injected with the 1,1,1,2-tetrachloroethane as an impurity. There was thus no detectable formation of trichloroethylene.

From the figures in table 1 it can be calculated that the combined trichloroethanol and trichloroacetic acid are equivalent to an average yield of 60 per cent of the dose metabolized, i. e. the difference between administered and expired tetrachloroethane. However, since small amounts of tetrachloroethane and its metabolites still remained in the organism after 3 days, this figure must be regarded as a minimum value.

Formation of trichloroacetic acid from 2,2,2-trichloroethanol. In order to study the possibility of trichloroethanol being the precursor of trichloroacetic acid in mice, this compound was determined in urine collected for 2 days after the administration of trichloroethanol. The dose was in the vicinity of the LD50. Determinations of trichloroacetic acid showed a mean excretion of 3.1 per cent (range 2.4–5.3) of the dose.

Table 1.

The excretion of unchanged 1,1,1,2-tetrachloroethane in expired air and *trichloroethanol and trichloroacetic acid in urine (including faeces) from mice receiving the chlorohydrocarbon by subcutaneous injection. Percentage of dose (molar).*

Dose g/kg	1,1,1,2-tetrachloroethane[a]				Trichloroethanol[b]				Trichloroacetic acid[b]				Total
	0–24 hrs	24–48 hrs	48–72 hrs	Sum	0–24 hrs	24–48 hrs	48–72 hrs	Sum	0–24 hrs	24–48 hrs	48–72 hrs	Sum	
1.2	47.9	4 8	1 3	54 0	17.9	6.3	0.7	24 9	1.6	1.1	0 6	3.3	82.2
1.3	27.1	1.1	1.0	29.2	32 3	10.8	0.0	43.1	3.2	2.7	0.1	6.0	78.3
1.5	18.7	2.2	0 2	21.1	31.8	15.0	2.2	49.0	4.2	2.7	0.2	7.1	77.2
1.8	37.9	1.4	1.1	40.4	27.0	0.2	0.2	27.2	2.2	1.6	0.4	4.2	71.8
1.9	57 2	3.5	0 9	61 6	15 5	1.8	0.1	17.4	1.1	0.5	0.1	1.7	80.7
2.0	48.4	4.1	0.5	53.0	20.8	4.4	0 4	25.6	0.7	0.4	0.0	1.1	79.7

[a] Each figure is a mean of 3 gas chromatographic determinations of a cumene solution.
[b] Each figure is a mean of 2 determinations.

REFERENCES

Allgén, L.-G., U. H. Lindberg & S. Ullberg: Vävnadsdistribution och metabolism för heminevrin (4-methyl-5-β-kloretyltiazol-etandisulfonat). *Nord. Psyk. Tidskr.* 1963, **17**, 13–19.

Bray, H. G., S. P. James & W. V. Thorpe: Metabolism of some ω-halogenoalkylbenzenes and related alcohols in the rabbit. *Biochem. J.* 1958, **70**, 570–579.

Bray, H. G., W. V. Thorpe & D. K. Vallance: The liberation of chloride ions from organic chloro compounds by tissue extracts. *Biochem. J.* 1952, **51**, 193–201.

Butler, T. C.: The metabolic fate of chloral hydrate. *J. Pharmacol.* 1948, **92**, 49–58.

Cabana, B. E. & P. K. Gessner: The kinetics of chloral hydrate metabolism in mice and the effect thereon of ethanol. *J. Pharmacol. Exp. Therap.* 1970, **174**, 260–275.

Daniel, J. W.: The metabolism of ^{36}Cl-labelled trichloroethylene and tetrachloroethylene in the rat. *Biochem. Pharmacol.* 1963, **12**, 795–802.

Defalque, R. J.: Pharmacology and toxicology of trichloroethylene. A critical review of the world literature. *Clin. Pharmacol. Therap.* 1961, **2**, 665–688.

Garrett, E. R. & H. J. Lambert: Gas chromatographic analysis of trichloroethanol, chloral hydrate, trichloroacetic acid, and trichloroethanol glucuronide. *J. Pharm. Sci.* 1966, **55**, 812–817.

Heppel, L. A. & V. T. Porterfield: Enzymatic dehalogenation of certain brominated and chlorinated compounds. *J. Biol. Chem.* 1948, **176**, 763–769.

Jondorf, W. R.: The metabolism of certain chlorinated hydrocarbons. Dissertation. University of London, 1956.

Jondorf, W. R., D. V. Parke & R. T. Williams: The metabolism of ^{14}C-hexachloroethane. *Biochem. J.* 1957, **65**, 14 p.

Knight, R. H. & L. Young: Biochemical studies of toxic agents. 11. The occurrence of premercapturic acids. *Biochem. J.* 1958, **70**, 111–119.

Kylin, B., H. Reichard, I. Sümegi & S. Yllner: Hepatotoxicity of inhaled trichloroethylene, tetrachloroethylene and chloroform. Single exposure. *Acta pharmacol et toxicol.* 1963, **20**, 16–26.

Marshall Jr., E. K. & A. H. Owens Jr.: Absorption, excretion and metabolic fate of chloral hydrate and trichloroethanol. *Bull. Johns Hopkins Hosp.* 1954, **95**, 1–18.

Mayer, F.: Zum Verhalten von Trichloracetat und anderen Halogenacetaten gegenüber Sulfhydryl- und Aminogruppen sowie einigen sekundären Pflanzenstoffen, *Biochem. Z.* 1957, **328**, 433–442.

Prins, H. J.: Notiz über eine Methode zur Darstellung von 1,1,2-Tri- und 1,1,1,2-Tetrachloraethan. *Receuil Trav. Chim. Pays-Bas* 1922, **45**, 80–81.

Seto, T. A. & M. O. Schultze: Isolation and structure of urochloralic acid from urine of calves fed trichloroethylene. *J. Am. Chem. Soc.* 1956a, **78**, 1616–1618.

Seto, T. A. & M. O. Schultze: Determination of trichloroethylene, trichloroacetic acid and trichloroethanol in urine. *Anal. Chem.* 1956b, **28**, 1625–1629.

Smith, G. F.: Trichloroethylene: A review. *Brit. J. industr. Med.* 1966, **23**, 249–262.

Widmark, E.: *Handbuch für biologische Arbeitsmethoden.* Berlin-Wien, 1932, *V:2,2*, p. 1935.

Williams, R. T.: *Detoxication mechanisms.* London 1959, p. 25.

Yllner, S.: The metabolism of pentachloroethane and unsymmetric tetrachloroethane. *Proc. XIV. Int. Congr. Occupat. Health*, Madrid, 1963, 825–827.

Yllner, S.: Metabolism of pentachloroethane in the mouse. *Acta pharmacol. et toxicol.* 1971a, **29**, 481–489.

Yllner, S.: Metabolism of chloroacetate-1-^{14}C in the mouse. *Acta pharmacol. et toxicol.* 1971b, **30**, in press.

Acta pharmacol. et toxicol. 1971, 29, 481–489.

From the National Institute of Occupational Health,
Stockholm, Sweden

Metabolism of Pentachloroethane in the Mouse

By

Sven Yllner

(Received July 28, 1970)

Abstract: Pentachloroethane was given subcutaneously to mice (1.1–1.8 g/kg) and the excretion was followed for 3 days. About one third of the dose (range 12–51 %) was expired unchanged The metabolized part was excreted mainly as trichloroethanol (16–32 % of the dose) and trichloroacetic acid (9–18 %) in the urine. The expired air also contained trichloroethylene (2–16 %) and tetrachloroethylene (3–9 %) indicating both dechlorination and dehydrochlorination of pentachloroethane. The latter reaction was found to be non-enzymatic. The metabolites were identified by paper chromatography, gas chromatography and mass spectrometry, and the Fujiwara reaction was used for the quantitative determination of trichloroacetic acid and of trichloroethanol after oxidation to this acid. At least part of the urinary metabolites must be formed via trichloroethylene and its metabolite chloral hydrate. The latter compound may also be formed from pentachloroethane by hydrolytic fission of carbon-chlorine bonds.

Key-words: Pentachloroethane, metabolism.

Pentachloroethane is an intermediary product in the manufacture of tetrachloroethylene. It has found practically no application as an industrial solvent and is reported to be a strong narcotic (von Oettingen 1955).

The present study, which demonstrates the fate of pentachloroethane in the organism, is part of an investigation of the metabolism of chloro derivatives of ethane. A preliminary report was published by Yllner (1963).

Materials and methods

Boiling points are uncorrected and measured at atmospheric pressure.

Pentachloroethane. A technical product was treated with dilute bicarbonate solution, washed with water and dried over calcium chloride. Repeated distillation *in vacuo* (10 mmHg) was performed. The boiling point of the final product was 162°. Gas chromatographic examination using columns nos. 1 and 3 (see below) disclosed about 0.5 per cent of impurities (probably mostly hexachloroethane).

2,2-Dichloroethanol was prepared according to SROOG & WOODBURN (1952). B. p. 146°.

The synthesis and purification of trichloroacetic acid methyl ester and the purification of 2,2,2-trichloroethanol are described elsewhere (YLLNER 1971).

Other chemicals used were trichloroethylene (analytical grade, Baker Chemicals), tetrachloroethylene (min. 99 per cent, Merck AG), 2-chloroethanol (analytical grade, Merck AG), chloroacetic acid (analytical grade, British Drug Houses, Ltd), dichloroacetic acid (technical product, 99 per cent) and trichloroacetic acid (analytical grade, Merck AG).

Animals. Female albino mice of the NMRI strain fed *ad libitum* on water and a standard diet (KYLIN *et al.* 1963) and weighing 20–30 g were used throughout the experiments.

Gas chromatography. Gas chromatography was performed with a Perkin-Elmer fractometer no. 116 equipped with a thermistor detector and a 5 mV recorder. The columns, (2-m aluminium tubes, ¼" inner diam.) contained the following stationary phases absorbed on 10 times their weight Chromosorb W (60–80 mesh):(1) Paraffin (m.p. 50–52°); (2) polyethylene glycol 1500; (3) octyl phthalate; (4) silicone SE 52; (5) tricresylphosphate. Carrier gas: helium.

Mass spectrometry. Metabolites were identified by means of combined gas chromatography and mass spectrometry with the LKB instrument no. 9000. Carrier gas: helium. The compounds were introduced as 0.1–0.5 per cent solutions in ether, and identities were established by comparison with mass spectra obtained from authentic samples by the same procedure. Separation of the exhaled compounds pentachloroethane, tri- and tetrachloroethylene was carried out with a column containing Apiezon L, 10 per cent on Chromosorb W, and a temperature gradient of 50 → 110°. Trichloroethanol and trichloroacetic acid methyl ester were separated on a column containing 10 per cent of polyethylene glycol 1500 on Cromosorb W, the alcohol at 145° and the ester at 70°.

Degradation of pentachloroethane in neutral aqueous solution.

1. *Determination of liberated chloride ions.* Pentachloroethane (0.20 ml, 1.7 mmol) was dissolved in ethanol (40 ml) and 0.2 M potassium phosphate buffer (60 ml, pH 7.0). The reaction mixture was incubated at 37°. To samples (5 ml) acidified with nitric acid, 0.01 M silver nitrate (5 ml) was added and the excess Ag^+ estimated by Volhard titration with 0.01 M potassium thiocyanate.

2. *Formation of tetrachloroethylene.* Samples (5 ml) of the above reaction mixture were added to hexane (1 ml) and water (100 ml). The mixture was shaken thoroughly and the hexane layer analysed by gas chromatography using column no. 4. References containing known percentage of pentachloroethane and tetrachloroethylene were used.

3. *Fujiwara test.* Samples of the reaction mixture were tested for chloral and/or trichloroethylene by the Fujiwara reaction as modified by SETO & SCHULTZE (1956).

Dosage and collection of metabolites. Pentachloroethane was administered by subcutaneous injection in the scapula region with a calibrated 50 μl Hamilton syringe. After dosing, the mouse was placed in an all-glass chamber from which the urine, faeces and expired metabolites were collected simultaneously and analysed daily. Each experiment was run for 3 days, during which time the mouse received 5 per cent glucose solution *ad libitum* but no food. The urine and faeces were analysed together.

The glass chamber was supplied with a constant slow current of dry air free of carbon dioxide. Volatile metabolites in the air leaving the chamber were collected by one of two trapping devices:

1. The air was dried, and volatile metabolites trapped in cumene at −80° as described elsewhere (YLLNER 1971). Gas chromatography was performed with columns

nos. 1, 3, 4 and 5. Since this examination did not exclude the presence of metabolites with the same retention times as cumene (with all columns the cumene peak appeared between those of tetrachloroethylene and pentachloroethane) the experiment was repeated with cumene replaced by ethanol. Pentachloroethane and tetrachloroethylene in the breath were trapped quantitatively by the cumene. When pentachloroethane was evaporated into the metabolism chamber without any animal present, examination of the cumene trap disclosed no tri- or tetrachloroethylene; this showed that none of these compounds was an artefact formed when the pentachloroethane vapour was passed through the drying unit.

2. For the determination of trichloroethylene which was not retained quantitatively by the cumene trap, a glass U-tube containing a 10 cm filling of silicone SE 30, 10 per cent on Chromosorb W, (kept at −80°) was used instead.

The same U-tube was used for trapping expired metabolites for identification by mass spectrometry. Volatile compounds absorbed in the tube were then transferred to 2 ml of ether at 0° by means of a current of air passed through the U-tube heated to 100°. The ether solution was examined by combined gas chromatography and mass spectrometry.

Determination of expired metabolites. Pentachloroethane and tetrachloroethylene in the cumene solutions were estimated by gas chromatography (column no. 3 at 110°) as described for 1,1,1,2-tetrachloroethane (YLLNER 1971). Trichloroethylene was determined by the following procedure: The U-tube with the trapped trichloroethylene (and other chlorohydrocarbons) was attached to the first column connection in the preheated gas chromatograph, and column no. 3 was attached to the second connection. When a constant temperature (90°) had been regained (1–2 min.) the carrier gas (helium), which passed first through the U-tube and then through the column, was turned on. The gas pressure immediately reached the previously adjusted level of 0.5 kg/cm². The trapped compounds were then rapidly conveyed by the gas stream into the chromatographic column. The reference solutions of trichloroethylene (in hexane) were injected with the U-tube still in the chromatograph. The amounts of trichloroethylene were calculated from the references and a calibration curve.

Analytical procedures for urinary metabolites.

1. *Paper chromatography.* Urinary metabolites from a mouse dosed with 20 µl of pentachloroethane were examined by paper chromatography. The procedures were the same as used previously with 1,1,1,2-tetrachloroethane (YLLNER 1971).

2. *Isolation and identification.* Urine from 40 mice each receiving 30 µl of pentachloroethane was collected for 48 hours. Assay of trichloroacetic acid (see below) gave a total of 69 mg. The urine was acidfied and extracted with ether and the ether extract was methylated with diazomethane (YLLNER 1971). Examination of the methylated product by gas chromatography (column no. 3) showed a main peak corresponding to methyl trichloroacetate. The product was finally examined by combined gas chromatography and mass spectrometry, and the peak corresponding to trichloroacetic acid methylester was found to give a mass spectrum identical with the authentic sample.

Urine (10 ml), obtained as described above, was heated with concentrated hydrochloric acid (5 ml) in a sealed glass ampoule at 100° for one hour. The pH was adjusted to about 5 and the solution extracted continuously with ether overnight. The ether [illegible] tion was dried over anhydrous sodium sulphate, evaporated to about 0.2 ml [illegible] examined for mono-, di- and trichloroethanol by gas chromatography [illegible] and 3). Only trichloroethanol was found, and this amounted to about 5 per [illegible] dose.

Urine, adjusted to pH 4.5 by an acetate buffer, was incubated with *β-glucuronidase* at 37° for 48 hours as described by DANIEL (1963) and the liberated trichloroethanol isolated (YLLNER 1971).

3. *Quantitative determination.* Trichloroacetic acid and trichloroethanol in the combined faeces and urine were determined as previously described (YLLNER 1971).

Results

In vitro experiments. When pentachloroethane was dissolved in a neutral buffer solution (by addition of alcohol) and incubated at 37°, chloride ions were liberated (fig. 1). In 24 hours there was a turnover of about 80 per cent, calculated as 1 Cl^-/C_2HCl_5. Gas chromatography showed that tetrachloroethylene was formed in a quantity equivalent to the liberation of chloride. The absence of any colouration with the Fujiwara reaction demonstrated that no chloral or trichloroethylene was formed.

Volatile metabolites. Gas chromatography of the cumene solution containing trapped volatile metabolites showed peaks with retention times equal not only to pentachloroethane but also to tri- and tetrachloroethylene. The identity of these compounds was established by mass spectrometry. Gas chromatographic examination (column no. 3) of metabolites trapped in ethanol instead of cumene disclosed no further metabolites.

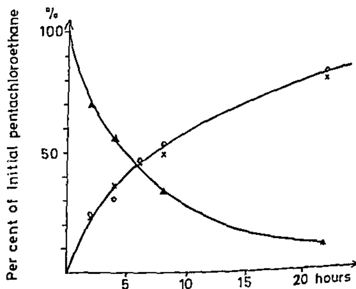


Fig. 1. Degradation of pentachloroethane in buffer solution (pH 7.0) at 37°.

△–△–△ pentachloroethane.
×–×–× tetrachloroethylene.
○–○–○ chloride calculated as 1 Cl^-/C_2HCl_5.

Urinary metabolites. Urine from a mouse dosed with pentachloroethane (20 µl) was examined by paper chromatography with 3 solvent systems. The results were similar to those obtained with 1,1,1,2-tetrachloroethane (YLLNER 1971). Trichloroacetic acid and trichloroethanol (conjugated) were the only metabolites found. Trichloroacetic acid and 2,2,2-trichloroethanol were isolated from the urine and identified by mass spectrometry.

Quantitative determinations of metabolites. The excreted amounts of identified metabolites from mice receiving pentachloroethane are shown in table 1. The mean amount of pentachloroethane expired unchanged was one third of the dose, but the range was large and there was a tendency for the relative amount to increase with the dose. Most of it was expired in the first 24 hours.

The amount of trichloroethylene expired varied even more than pentachloroethane. However, in this case the dose range was not large and there was no correlation found between the amount of trichloroethylene excreted and the dose of pentachloroethane. The excretion of the other volatile metabolite, tetrachloroethylene, was far more constant but also did not show any definite correlation to the dose.

Table 1.

Excreted amounts (mean values) of pentachloroethane and its metabolites as a percentage of the injected dose Pentachloroethane, tetrachloroethylene, trichloroethanol and trichloroacetic acid were determined simultaneously while trichloroethylene was determined in separate runs.

The figures in square brackets denote the number of mice examined and those in parantheses the range of values. In the case of C_2HCl_5 and C_2Cl_4 each value is the mean of at least 3 determinations, and for CCl_3CH_2OH and CCl_3COOH the mean of 2 determinations; for C_2HCl_3 each value corresponds to one determination.

	Dose C_2HCl_5 g/kg	0–24 hrs	24–48 hrs	48–72 hrs	72–96 hrs	Total
C_2HCl_5	1.1–1.8	26 7 [7] (8–48)	4.5 [7] (2–8)	0.6 [7] (0–1.4)	–	31.8 (12–51)
C_2Cl_4	1 1–1 8	3.5 [6] (1.6–3.9)	3 3 [6] (1.1–3 8)	0.6 [6] (0–1.2)	–	7.4 (3–9)
C_2HCl_3	1.0–1 4	6.0 [11] (1.4–14)	2.4 [7] (0 1–5.4)	0.1 [4] (0–0 4)	–	8.5 (2–16)
CCl_3CH_2OH	1.1–1.8	12.4 [6] (7–17)	9 4 [6] (5–14)	1.7 [6] (0.2–3)	–	23.5 (16–32)
CCl_3COOH	1.1–1.8	6 3 [6] (3–9)	5 8 [6] (5–6)	2.5 [5] (1–4)	1.5 [4] (1–2)	-

About 40 per cent of the dose was determined as trichloroethanol and trichloroacetic acid. A calculation of pentachloroethane and its metabolites in the expired air shows that trichloroethanol and trichloroacetic acid constitute at least three quarters of the metabolites in the urine and faeces. The excretion of trichloroacetic acid is known to be rather slow, and the compound can be detected in the urine weeks after administration (AHLMARK & FORSSMAN 1951; SOUČEK & VLACHOVÁ 1960; BARTONIČEK 1962). Some additional trichloroacetic acid would therefore remain in the animal after the observation period. Thus, the urinary metabolites consist largely of trichloroethanol and trichloroacetic acid.

Metabolites of trichloroethylene. Since trichloroethylene was found to be a metabolite of pentachloroethane it was considered of interest to examine the excretion of trichloroacetic acid and trichloroethanol after the administration of trichloroethylene.

Each of 5 mice was given trichloroethylene (20 µl) by subcutaneous injection and the urine was collected separately. Determinations of trichloroacetic acid and trichloroethanol excreted during 48 hours were performed. The mean yield of trichloroacetic acid was 4.8 per cent (range 3.4–6.7) and that of trichloroethanol 16.3 per cent (range 13.5–18.9) of the dose.

Discussion

On the basis of the results the metabolic pathways of pentachloroethane shown in fig. 2 are suggested.

In their study of the liberation of chloride ions from organic chloro compounds in neutral buffer solution BRAY *et al.* (1952) found a pentachloroethane degradation of 13 per cent in 24 hours (expressed as 1 Cl^-/mol). No reaction products other than chloride were determined. When the experiment was repeated in this study under similar conditions a considerably higher reaction rate was found; about 80 per cent of the pentachloroethane reacted in 24 hours, and gas chromatography showed an equivalent formation of tetrachloroethylene. No trichloroethylene or chloral was formed. Thus, the non-enzymatic dehydrochlorination of pentachloroethane at pH 7 is con-

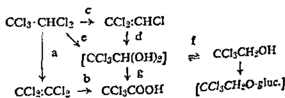


Fig. 2. Suggested metabolic pathways of pentachloroethane. The compounds in brackets were not isolated.

siderable and, as would be expected, tetrachloroethylene was also found as a metabolite. It was also shown by FOWLER (1969) that tetrachloroethylene is a metabolite of pentachloroethane given orally to sheep. In a study by YLLNER (1961) in which mice were exposed to the vapour of ^{14}C-tetrachloroethylene, 70 per cent of the absorbed amount was expired unchanged. Trichloroacetic acid was the main metabolite formed. The formation of this acid was confirmed by DANIEL (1963), who administered ^{36}Cl-labelled tetrachloroethylene to rats. These findings are consistent with reactions *a* and *b* in fig. 2. When pentachloroethane was administered to mice parenterally, the average amount of tetrachloroethylene expired was not more than 7 per cent of the dose, while 16 per cent was excreted as trichloroacetic acid. Most of the acid would therefore seem to have some other precursor than tetrachloroethylene.

The fact that, in addition to tetrachloroethylene, about the same amount of trichloroethylene was found in the expired air indicates that dechlorination too, is a primary metabolic reaction of pentachloroethane (reaction *c*). This type of reaction was found by JONDORF (1956) to occur in the metabolism of hexachloroethane in the rabbit; some 12 per cent of the hexachloroethane dose was excreted as tetrachloroethylene. Similar observation was made by FOWLER (1969) on the hexachloroethane metabolism in the sheep.

The metabolism of trichloroethylene has been extensively studied, and the comprehensive literature reviewed by DEFALQUE (1961) and SMITH (1966). Trichloroethylene is oxidized *in vivo* with the formation of trichloroacetic acid and trichloroethanol, and chloral hydrate is an intermediary metabolite. The conversion of trichloroethylene to chloral hydrate (reaction *d*) was shown by LEIBMAN (1965) and BYNINGTON & LEIBMAN (1965) to take place in liver microsomes of the rat, rabbit and dog in a reaction requiring the presence of NADPH and oxygen.

The formation of chloral hydrate in the metabolism of trichloroethylene indicates a molecular rearrangement with the migration of chlorine. This is consistent with DANIEL'S (1963) observation that ^{36}Cl-trichloroethylene is metabolized to trichloroacetic acid and trichloroethanol with an unchanged specific activity, and no exchange of chloride with the body chloride pool.

Depending on the species, dose and mode of administration there is a great variation in the amount of trichloroethylene metabolized (FORSSMAN & HOLMQUIST 1953; BARTONIČEK & TEISINGER 1962; DANIEL 1963). It is therefore impossible to calculate what portion of the urinary metabolites of pentachloroethane was actually formed via trichloroethylene. It may be the precursor of all the trichloroethanol and most of the trichloroacetic acid, but the ratio between the exhaled trichloroethylene and the combined [illegible] trichloroethanol and trichloroacetic acid (about 1:5) indicates an ac[illegible] metabolic pathway of pentachloroethane, with the formation of [illegible] ethanol and trichloroacetic acid. In the present study, an a[illegible]

21 per cent of the trichloroethylene injected in the mice was excreted as trichloroacetic acid and trichloroethanol in 48 hours.

The fact that, as reported by YLLNER (1971), 1,1,1,2-tetrachloroethane is metabolized to trichloroethanol suggests hydrolysis of a carbon-chlorine bond. If this is also attributable to pentachloroethane metabolism it would result in the direct formation of chloral hydrate (reaction *e*). A similar reaction has been observed in the case of the anaesthetic halothane. When ^{14}C- or ^{36}Cl-labelled preparations of 1,1,1-trifluoro-2-bromo-2-chloroethane (halothane) were used this compound was found to be partly dehalogenated *in vivo* and excreted as trifluoroacetic acid and the glucuronide of trifluoroethanol (VAN DYKE & CHENOWETH 1965; STIER & ALTER 1966; VAN DYKE 1966). The cleavage of the carbon-halogen bond was found to be catalyzed by enzymes in the microsomes, and the reaction required the presence of NADH and oxygen.

The reduction of chloral hydrate to trichloroethanol (reaction *f*) was observed *in vitro* by BUTLER (1949) to occur in most tissues of the dog and rat under aerobic conditions. OWENS & MARSHALL (1955) found that the reduction of chloral hydrate was catalysed *in vitro* by crystalline horse liver alcohol dehydrogenase with DPNH (= NADH) as the reductant. Although trichloroethanol is oxidized *in vivo* to trichloroacetic acid these investigators detected no oxidation *in vitro* in the presence of liver or yeast alcohol dehydrogenase and DPN (= NAD). However, the oxidation *in vivo* was completely prevented by previous and concomitant administration of ethanol. Thus, ethanol and trichloroethanol seem to share common pathways of metabolism. The kinetics of chloral hydrate metabolism in mice and the effect thereon of ethanol has recently been studied by CABANA & GESSNER (1970).

The oxidation of chloral hydrate (reaction *g*), was found by BUTLER (1949) to occur only in liver and kidney preparations. An NAD-dependent system that catalyses the oxidation of chloral hydrate to trichloroacetic acid was isolated from rabbit liver by COOPER & FRIEDMAN (1958). Remarkably enough, no naturally occurring substrate for this enzyme was found.

REFERENCES

Ahlmark, A. & S. Forssman: Evaluating trichloroethylene exposures by urinalyses for trichloroacetic acid. *Arch. Ind. Hyg. Occupat. Med.* 1951, 3, 386–398.

Bartoníček, V.: Metabolism and excretion of trichloroethylene after inhalation by human subjects. *Brit. J. ind. Med.* 1962, 19, 134–141.

Bartoníček, V. & J. Teisinger: Effect of tetraethyl thiuram disulphide (disulfiram) on metabolism of trichloroethylene in man. *Brit. J. ind. Med.* 1962, 19, 216–221.

Bray, H. G., W. V. Thorpe & D. K. Vallance: The liberation of chloride ions from organic chloro compounds by tissue extracts. *Biochem. J.* 1952, 51, 193–201.

Butler, T. C.: Reduction and oxidation of chloral hydrate by isolated tissues *in vitro*. *J. Pharmacol.* 1949, **95**, 360–362.
Byington, K. H. & K. C. Leibman: Metabolism of trichloroethylene in liver microsomes. II. Identification of the reaction product as chloral hydrate. *Mol. Pharmacol.* 1965, **1**, 247–254.
Cabana, E. B. & P. K. Gessner: The kinetics of chloral hydrate metabolism in mice and the effect thereon of ethanol. *J. Pharmacol. Exp Therap.* 1970, **174**, 260–275.
Cooper, J. R. & P. J. Friedman: The enzymic oxidation of chloral hydrate to trichloroacetic acid. *Biochem. Pharmacol.* 1958, **1**, 76–82
Daniel, J. W.: The metabolism of ^{36}Cl-labelled trichloroethylene and tetrachloroethylene in the rat. *Biochem. Pharmacol.* 1963, **12**, 795–802.
Defalque, R. J.: Pharmacology and toxicology of trichloroethylene. A critical review of the world literature. *Clin. Pharmacol. Therap.* 1961, **2**, 665–688.
Forssman, S. & C.-E. Holmquist: The relation between inhaled and exhaled trichloroethylene and trichloroacetic acid excreted in the urine of rats exposed to trichloroethylene. *Acta pharmacol. et toxicol.* 1953, **9**, 235–244.
Fowler, J. S. L.: Some hepatotoxic actions of hexachloroethane and its metabolites in sheep. *Brit. J. Pharmacol.* 1969, **35**, 530–542
Jondorf, W. R.: The metabolism of certain chlorinated hydrocarbons. Dissertation, University of London, 1956.
Kylin, B., H. Reichard, L. Sümegi and S. Yllner: Hepatotoxicity of inhaled trichloroethylene, tetrachloroethylene and chloroform. Single exposure *Acta pharmacol. et toxicol.* 1963, **20**, 16–26.
Leibman, K. C.: Metabolism of trichloroethylene in liver microsomes. I Characteristics of the reaction. *Mol Pharmacol.* 1965, **1**, 239–246.
von Oettingen, W. F.: The halogenated aliphatic, olefinic, cyclic, aromatic and aliphatic-aromatic hydrocarbons including the halogenated insecticides, their toxicity and potential dangers *Public Health Service, Publication No. 414.* Washington 1955.
Owens, Jr. A. H. & E. K Marshall, Jr.: A comparison of the metabolism of ethanol and trichloroethanol. *Bull Johns Hopkins Hosp.* 1955, **97**, 395–404
Seto, T. A. & M. O. Schultze: Determination of trichloroethylene, trichloroacetic acid and trichloroethanol in urine. *Anal Chem* 1956, **28**, 1625–1629.
Smith, G. F.: Trichloroethylene. A review *Brit J ind Med.* 1966, **23**, 249–262.
Souček, B. & D. Vlachová: Excretion of trichloroethylene metabolites in human urine. *Brit. J. ind. Med* 1960, **17**, 60–64
Sroog, C. E & H M Woodburn: 2,2-Dichloroethanol *Org. Synthesis* 1952, **32**, 46–48.
Stier, A. & H. Alter· Stoffwechselprodukte des Halothane im Urin. *Anaesthesist* 1966, **15**, 154–155.
Van Dyke, R. A.: Metabolism of volatile anaesthetics. III. Induction of microsomal dechlorinating and ether-cleaving enzymes. *J Pharmacol. Exp. Therap.* 1966, **154**, 364–369.
Van Dyke, R. A. & M. B Chenoweth: The metabolism of volatile anaesthetics. *In vitro* metabolism of methoxyflurane and halothane in rat liver slices and cell fractions *Biochem. Pharmacol.* 1965, **14**, 603–609.
Yllner, S.: Urinary metabolites of ^{14}C-tetrachloroethylene in mice. *Nature* 1961, **191**, 820.
Yllner, S.: The metabolism of pentachloroethane and unsymmetric tetrachloroethane. *Proc. XIV. Int Congr Occupat. Health.* Madrid, 1963, 825–827.
Yllner, S.: Metabolism of 1, 1, 1, 2-tetrachloroethane in the mouse. *Acta p[illegible] toxicol.* 1971, **29**, [illegible]

Acta pharmacol. et toxicol. 1971, 29, 490–498.

From the National Veterinary Institute, Oslo, Norway

Methaemoglobin Formation *in Vitro* by 6-Amino Metabolites of DNOC and DNBP

By

Arne Froslie

(Received September 4, 1970)

Abstract: The six compounds DNOC, DNBP, 6-ANOC, 6-ANBP, 6-AcANOC and 6-AcANBP were investigated for their methaemoglobin-forming effect on bovine erythrocytes *in vitro*, and also, for comparative reasons, on erythrocytes from horse, dog, pig and man. Only the amino-nitrophenols showed any methaemoglobin-forming effect. The activity was somewhat different for the different species, the haemoglobin in dog erythrocytes in particular being more easily oxidized than the haemoglobin in erythrocytes from the other species. Differences were also recorded between the two amino-nitrophenols, 6-ANOC being more active than 6-ANBP in the bovine erythrocytes, while 6-ANBP was more active in the pig and horse erythrocytes. In the dog and man there was a little difference between the two substances. The results are discussed in relation to the dinitrophenols' comparative toxicology, in particular in relation to the ruminants' special position as regards the metabolism and the toxicity of these substances.

Key-words: Methaemoglobin - dinitrophenols, metabolism.

Methaemoglobinemia due to 4,6-dinitrophenol poisoning in ruminants has been demonstrated by FRØSLIE & KARLOG (1970), as intraruminal application of 2-(1-methyl-n-propyl)-4,6-dinitrophenol (DNBP) in cattle led to the formation of considerable amounts of methaemoglobin, while the effect of 2-methyl-4,6-dinitrophenol (DNOC) was weaker. Because methaemoglobinemia in connection with dinitrophenol poisoning is observed only in ruminants, these investigators considered it likely that the absorption of amino-metabolites, which are rapidly formed in the rumen, was responsible for the formation of methaemoglobin.

To elucidate this hypothesis I investigated the effect of the two dinitrophenols, as well as their 6-amino-, and their 6-acetamido derivatives on erythrocytes *in vitro*. Since the ruminants are unique as regards both the metabolism and the toxicity of dinitrophenols (HARVEY 1958; NEGHERBON 1959; RADELEFF & BUSHLAND 1960; FROSLIE & KARLOG 1970) erythrocytes from other species have, for comparative reasons, also been investigated.

Materials and methods

2-methyl-4,6-dinitrophenol (4,6-dinitro-o-cresol; DNOC).
2-(1-methyl-n-propyl)-4,6-dinitrophenol (4,6-dinitro-2-sec.-butylphenol; DNBP; DINOSEB).
2-methyl-4-nitro-6-aminophenol (6-amino-4-nitro-o-cresol; 6-ANOC).
2-(1-methyl-n-propyl)-4-nitro-6-aminophenol (6-amino-4-nitro-2-sec.-butylphenol; 6-ANBP).
These substances were the same as those used by FROSLIE & KARLOG (1970).
2-methyl-4-nitro-6-acetamidophenol (6-acetamido-4-nitro-o-cresol; 6-AcANOC).
2-(1-methyl-n-propyl)-4-nitro-6-acetamidophenol (6-acetamido-4-nitro-2-sec.-butylphenol; 6-AcANBP).
These two substances were made from the corresponding amino compounds by N-acetylation according to the method described by VOGEL (1958). Their melting points were 225–228° and 145–147°, respectively.

Samples of venous blood from healthy cattle, horses, dogs, swine and man were pooled, sodium citrate being added as an anticoagulant, and the samples centrifuged. The erythrocytes were washed three times with physiological saline and once with a buffer consisting of 9 parts physiological saline and 1 part 6.7×10^{-2} M phosphate-citrate buffer, pH 7.4. The haemoglobin content was adjusted to a given level. The test substances, which were dissolved in the same buffer-saline solution, and the erythrocytes were covered with parafilm in test tubes, and incubated in a water bath at $39 \pm 1°$. Samples for methamoglobin determination were taken out at definite intervals.

Analytical methods.
The haemoglobin concentrations were determined by the cyanmethaemoglobin method, and the concentrations of methaemoglobin determined as described in STEWART & STOLMAN (1961).

Results

Effect of DNOC, DNBP, 6-AcANOC and 6-AcANBP on bovine erythrocytes.

DNOC, DNBP, 6-AcANOC and 6-AcANBP at concentrations of 3 and 5×10^{-4} M (for DNPB 1.5 and 3×10^{-4} M on account of its solubility) were incubated for 6 hours with bovine erythrocytes. The haemoglobin concentration was 3×10^{-3} M (5 g/100 ml).

In none of the experiments was any methaemoglobin formation noted.

Effect of 6-ANOC and 6-ANBP on bovine erythrocytes.

Bovine erythrocytes, with the same haemoglobin concentration as in the first experiments, were incubated with 6-ANOC and 6-ANBP at concentrations of 1, 2, 3, 4 and 5×10^{-4} M. The results are shown in figs. 1, 2 and 3.

From fig. 1 it is seen that both substances *in vitro* are active meth...

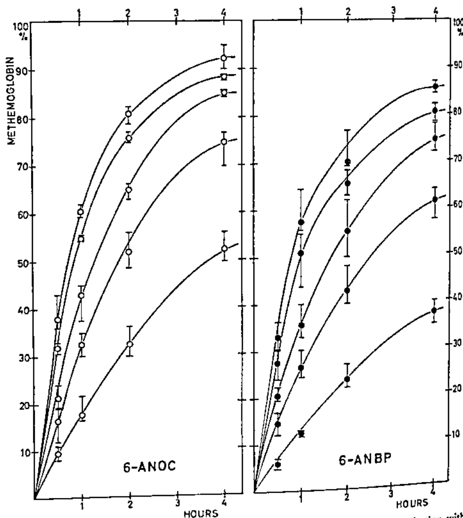


Fig. 1. Methaemoglobin formation *in vitro* in bovine erythrocytes after incubation with 6-ANOC and 6-ANBP at concentrations of 1, 2, 3, 4 and 5 $\times 10^{-4}$ M, the lowest curves: 1 $\times 10^{-4}$ M, the next: 2 $\times 10^{-4}$ M etc. The haemoglobin concentration was 3 $\times 10^{-3}$ M. The curves indicate the mean values and the distribution in three series of experiments.

bin-forming agents. After 2 hours' incubation, concentrations of 1 $\times 10^{-4}$ M result in about 30% methaemoglobin, while concentrations of 5 $\times 10^{-4}$ M result in about 80% (of total haemoglobin). The controls always showed less than 1 – 2%.

There is a marked tendency for 6-ANOC to have a more marked effect than 6-ANBP. This is also seen in fig. 2 and 3, where the results are pre-

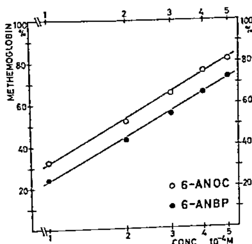


Fig. 2. Methaemoglobin formation *in vitro* in bovine erythrocytes after incubation with 6-ANOC and 6-ANBP for 2 hours at concentrations from 1 to 5 × 10^{-4} M. The curves show the relation between log concentration and amount of methaemoglobin. The same experiments as in fig. 1.

sented in a semilogarithmic scale. It is seen that there is a linearity between log dose and effect on haemoglobin (fig. 2) and between log incubation time and methaemoglobin percentage (fig. 3).

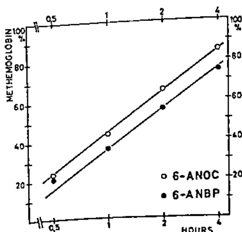


Fig. 3. Methaemoglobin formation *in vitro* in bovine erythrocytes after incubation with 6-ANOC and 6-ANBP at concentration of 3 × 10^{-4} M. The curves show the relati between log incubation time and amount of methaemoglobin. The same as in fig. 1.

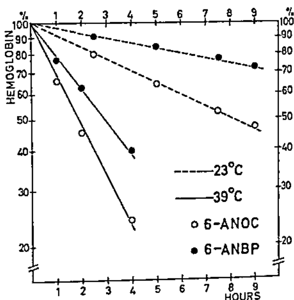


Fig. 4. Methaemoglobin formation *in vitro* in bovine erythrocytes after incubation with 6-ANOC and 6-ANBP at concentrations of 1.9 and 2.1×10^{-4} M, respectively, at 23° and 39°. The haemoglobin concentration was 3×10^{-3} M. The curves show the relation between incubation time and log remaining haemoglobin (100 % ÷ per cent methaemoglobin) at 23° and 39°, and indicate the mean values of 4 series of experiments each with 3 samples.

There is also a linearity between log remaining haemoglobin (100 % ÷ per cent methaemoglobin) and incubation time, as seen in fig. 4. Here the results of experiments comparing reaction velocities are presented in a semilogarithmic scale. Identical suspensions of bovine erythrocytes were incubated simultaneously with 6-ANOC and 6-ANBP at (approximately) identical concentrations (1.9 and 2.1×10^{-4} M, respectively). It is seen that there is a distinct difference in the reaction velocity for the two substances. After 2 hours' incubation the difference in methaemoglobin concentration was 16% (of total haemoglobin), and according to Student's t-test, this difference is significant ($P < 0.001$). For a further study of the reaction velocities, this experiment was also performed at room temperature (about 23°). Providing that the reaction velocities are temperature dependent, any difference in the velocity should be greater when the temperature is lowered. The upper curves in fig. 4 show that this is the case, and thus confirms that 6-ANOC *in vitro* is a stronger methaemoglobin-forming agent in bovine erythrocytes than 6-ANBP.

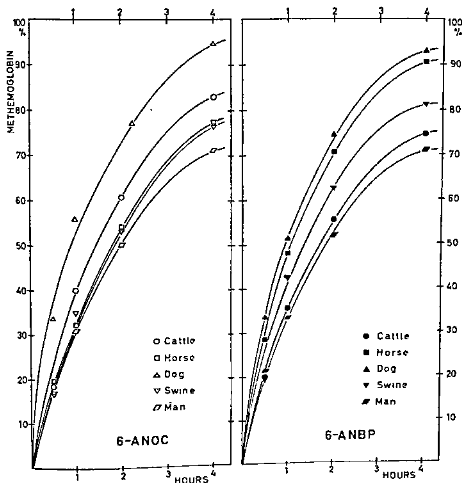


Fig. 5. Methaemoglobin formation *in vitro* in erythrocytes from cattle, horse, dog, swine and man after incubation with 6-ANOC and 6-ANBP at concentrations of 2.5 and 2.6 × 10-4 M, respectively. The haemoglobin concentration was 5 × 10-3 M. The curves indicate the mean values of 4 series of experiments each with 3 samples.

Comparative experiments to measure the methaemoglobin-forming activities of 6-ANOC and 6-ANBP in erythrocytes from cattle, horses, dogs, swine and man.

In these experiments haemoglobin concentrations of 5×10^{-3} M were used, and the concentrations of 6-ANOC and 6-ANBP were 2.5 and 2.6×10^{-4} M, respectively. The two series were incubated simultaneously under [illegible] cal conditions with identical suspensions of the respective erythrocytes results are shown in fig. 5.

It is seen from the curves that there is no essential difference

different erythrocytes, but there are certain quantitative differences. It is particularly clear that the haemoglobin in dog erythrocytes is more easily oxidized by 6-ANOC and 6-ANBP than the haemoglobin in the other erythrocytes, with one exception: The haemoglobin in horse erythrocytes is oxidized almost as readily by 6-ANBP as the haemoglobin in dog erythrocytes.

If one considers the amount of methaemoglobin present after 4 hours' incubation, and compares the respective effects of 6-ANOC and 6-ANBP on erythrocytes from the same species, it is clear that 6-ANOC is a stronger methaemoglobin-forming agent in bovine erythrocytes than 6-ANBP, with a difference of 8 % (of total haemoglobin). For erythrocytes from swine and particularly horses the position is the opposite, 6-ANBP being a more potent methaemoglobin-forming agent than 6-ANOC, while there is little difference in the erythrocytes from dogs and man. For the horse the difference after 4 hours' incubation is 22 %, while for the pig it is 7 %.

If one compares the different erythrocytes for haemoglobin conversion with each substance in turn, one sees that for 6-ANOC the activity falls in the following order: dog, cattle, horse, pig and man, with differences from cattle after 4 hours' incubation of: +15, 0, ÷5, ÷6 and ÷11 %, respectively. For 6-ANBP the corresponding order is: dog, horse, pig, cattle and man, with corresponding differences of: +19, +16, +9, 0 and ÷4 %. It is seen that the dog tops both activity series by a large margin, while human erythrocytes appear to be the least readily oxidized. Furthermore it is seen that the order of activity is different for the two substances. According to Student's t-test the differences reported are significant ($P < 0.001$).

Discussion

The majority of aminophenols are known to be active methaemoglobin-forming agents, and in man they are among the most common methaemoglobin-forming agents *in vivo* (Reviewed by Bodansky 1951; Uehleke 1964 and 1969; Kiese 1965 and 1966). The experiments reported here show that 6-ANOC and 6-ANBP also belong to the group of methaemoglobin-forming aminophenols.

Mayer & Vlès (1932) investigated the methaemoglobin-forming activity of dinitrophenols and their amino-nitro derivatives. They found that 2,4-dinitrophenol (DNP) had no effect *in vitro*, while 3,6-dinitrophenol had some effect. The amino-nitrophenols were the most active, and of these 2-amino-4-nitrophenol (2-ANP) was decidedly the most active. The present investigation confirms that the substituted meta-dinitrophenols DNOC and DNBP also do not oxidize haemoglobin *in vitro*, while the corresponding 6-amino-4-nitrophenols have the same effect as 2-ANP. Thus the essential effect of

these compounds on haemaglobin *in vitro* is analogous for these three dinitrophenols and their 6-amino derivatives.

Species differences in the reaction on methaemoglobin-forming agents can, apart from the activity of the reductase systems and the metabolism of the substances, be due to the response of the erythrocytes to the active agent. Thus KIESE & PEKIS (1964) found that species differences in methaemoglobinformation caused by *p*-aminophenol can be due to the activity of the substance in the erythrocytes themselves. They found that *p*-aminophenol *in vitro* was 15 times more active in erythrocytes obtained from the dog and cat than in erythrocytes obtained from cattle in experiments at room temperature.

The aminophenols investigated here also show species differences, and as with *p*-aminophenol, the erythrocytes from the dog are the most sensitive. Whether these differences are of significance for the actual comparative toxicology of these substances cannot be assessed from these experiments, but the results presented here do not support the assumption that such differences would explain the methaemoglobinemia in connection with dinitrophenol poisoning in ruminants. In the *in vitro* experiments cattle differ little from the other species, and this therefore indicates that the extensive production of amino derivatives in the rumen is a special factor with regard to ruminants.

The observation that 6-ANOC *in vitro* is a stronger methaemoglobinforming agent in bovine erythrocytes than 6-ANBP does not immediately appear consistent with FRØSLIE & KARLOG's findings *in vivo* (1970). They found DNBP to be a more potent methaemoglobin-forming agent in cattle than DNOC, but since these investigators found that 6-ANBP occurred in the blood plasma in an unconjugated form, whereas 6-ANOC was conjugated, probably acetylated, this discrepancy is easily explained. Acetylation is the most common detoxication mechanism for aromatic amines, and as UEHLEKE (1964) points out, acetylation has a decisive influence on their methaemoglobin-forming effect *in vivo*. The two acetamido derivatives showed, in accordance with this, no methaemoglobin-forming activity. Thus it is possible that an extensive conjugation of 6-ANOC in the organism inactivates this potent methaemoglobin-forming agent, while the slower metabolism of 6-ANBP leaves its effect unimpaired.

This supposition requires further from *in vivo* investigations, and it is also necessary to determine the significance of the diaminophenols, i. e. the main metabolites from the ruminal metabolism of DNOC and DNBP (FRØSLIE & KARLOG 1970), as methaemoglobin-forming agents *in vivo*. The diaminophenols have not yet been demonstrated in the blood plasma, but they belong to a group of compounds known to be potent methaemoglobin forming agents (KIESE 1966).

Acknowledgements

Special thanks are given to lic. pharm. Ole Karlog and Professor dr. med. vet. Sv. Dalgaard-Mikkelsen, the Institute of Pharmacology and Toxicology, Royal Veterinary and Agricultural University, Copenhagen, Denmark, for much advice and help.

This study was partly carried out at the National Veterinary Institute, Stockholm, Sweden, with financial support from a fellowship granted by the Swedish Institute, Stockholm, Sweden.

REFERENCES

Bodansky, D.: Methemoglobinemia and methemoglobin-producing compounds. *Pharmacol. Rev.* 1951, 3, 144–196.

Frøslie, A. & O. Karlog: Ruminal metabolism of DNOC and DNBP. *Acta vet. scand.* 1970, 11, 114–132.

Harvey, D. G.: Some aspects of the metabolism of 4,6-dinitro-o-cresol (DNC) by the ruminant. *J. comp. Path.* 1958, 68, 54–63.

Kiese, M.: Relationship of drug metabolism to methemoglobin formation. *Ann. N. Y. Acad. Sci.* 1965, 123, 141–155.

Kiese, M.: The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. *Pharmacol. Rev.* 1966, 18, 1091–1161.

Kiese, M. & M. Pekis: The reaction of *p*-aminophenol with hemoglobin and oxygen *in vivo* and *in vitro*. *Arch. exp. Path. Pharmakol.* 1964, 246, 413–420.

Mayer, A. & F. Vlès: Études sur l'action pharmacologique du dinitrophénol 1-2-4 et des phénols nitrés IX. – Action des phénols nitrés sur l'hémoglobine. *Ann. Physiol. Physicochim. biol.* 1932, 8, 176–194.

Negherbon, W. O.: Insecticides. In: *Handbook of toxicology*. Vol. III. W. B. Saunders, London 1959, p. 289–297.

.adeleff, R. D. & R. C. Bushland: The toxicity of pesticides for livestock. *U. S. Department of Agriculture* 1960, ARS 20-9, 134–160.

tewart, C. P. & A. Stolman: *Toxicology. Mechanisms and analystical methods*, vol. II. Acad. Press, London 1961, p. 788–789.

ehleke, H.: Biologische Oxydation und Reduktion am Stickstoff aromatischer Amino- und Nitroderivate und ihre Folgen für den Organismus. *Fortschr. Arzneimittelforsch.* 1964, 8, 195–260.

ehleke, H.: Toxikologische Aspekte der N-Hydroxylierung aromatischer Amine. *Arch. exp. Path. Pharmakol.* 1969, 263, 106–120.

Vogel, A. J.: *Elementary Practical Organic Chemistry*. Longmans, London 1958, p. 238.

Acta pharmacol. et toxicol. 1971, 29, 499–512.

From the National Institute of Occupational Health,
Stockholm, Sweden

Metabolism of 1,1,2,2-Tetrachloroethane-^{14}C in the Mouse

By

Sven Yllner

(Received July 28, 1970)

Abstract: Labelled tetrachloroethane was synthesized by addition of chlorine to ^{14}C-acetylene. The product (0.51 µci/mg) was injected intraperitoneally into female albino mice (dose 0.21–0.32 g/kg) and the elimination of radioactivity was followed for 3 days. Half of the dose (45–61 %) was expired as carbon dioxide and 28 % (range 23–34) of the activity was excreted with the urine About 16 % of the dose still remained in the animal and less than 4 % was expired unchanged. By paper chromatography and isotope dilution analysis, the urine was found to contain dichloroacetic acid, trichloroacetic acid, trichloroethanol, oxalic acid and small amounts of glyoxylic acid and urea Half of the urinary activity was not accounted for. On simultaneous injection of benzoate considerable amounts of labelled hippuric acid, about 20 % of the dose, were excreted. It is suggested that the metabolism of tetrachloroethane takes place mainly via a stagewise hydrolytic fission of carbon-chlorine bonds and oxidation to give first dichloroacetic acid and then glyoxylic acid. With part of the tetrachloroethane, a non-enzymic dehydrochlorination occurs with the formation of trichloroethylene which was also found in the breath. It is suggested that trichloroethylene is the precursor of the trichloroethanol and trichloroacetic acid found in urine.

Key-words: Tetrachloroethane – metabolism.

In their study of the pulmonary absorption of vapours of 1,1,2,2-tetrachloroethane LEHMANN & HASEGAWA (1910) found that about 80 per cent was retained in the body. According to GASQ's (1936) observation namely that 24 hours after exposure, the liver was the only organ still containing traces of tetrachloroethane, there would appear to be an extensive degradation *in vivo*. As regards the metabolic fate of this compound in the rat, guinea pig, dog and man, BARRETT *et al.* (1939) found no definite evidence that trichloroethylene is formed or that trichloroacetic acid is excreted. Since it was found that the urine of a man poisoned by tetrachloroethane contained an abnormal level of oxalate (LILLIMAN 1949) it was inferred that tetrachloroethane is metabolized by hydrolysis and subsequent oxidation. A preliminary report of the present study has been published (YLLNER 1966).

Materials and methods

All melting and boiling points are uncorrected and measured at atmospheric pressure.

1,1,2,2-Tetrachloroethane (puriss, min. 99 per cent, b. p. 146°). The product was examined by gas chromatography (see below) using TD (column nos. 1 and 2) and ECD (column no. 3). About 0.3 per cent of tetrachloroethylene but no trichloroethylene was found.

1,1,2,2-Tetrachloroethane-^{14}C. ^{14}C-Labelled tetrachloroethane was synthetized by McKinney & Pearce (1960) by thermal decomposition of the complex between labelled acetylene and antimony pentachloride. Yllner (1961) prepared ^{14}C-tetrachloroethane by adding chlorine to acetylene in the presence of anhydrous ferric chloride, a method based on the industrial synthesis of tetrachloroethane.

Acetylene-^{14}C (1 mci at 5 mci/μmol, The Radiochemical Centre, Amersham) (5.2 mg) was condensed on a high-vacuum line into an evacuated tube containing about 10 mg of freshly prepared anhydrous ferric chloride, the tube being fitted with a tap and cooled in liquid air. Dry chlorine (36.5 mg i. e. 2½ times the acetylene on a molar base) was condensed into the tube, which was closed and removed from the vacuum line, and the contents were allowed to reach and react at room temperature. After 18 hrs the reaction tube was re-attached to the vacuum line and cooled in an acetone/dry-ice bath. The tap was opened and the excess chlorine condensed into a liquid-air cooled flask containing a little potassium iodide solution. This flask was then allowed to reach room temperature and the iodine liberated was titrated with 0.1 N sodium thiosulphate, 2.34 ml was required, which is equivalent to 8.3 mg of chlorine. Thus, 28.2 mg of chlorine had reacted, which is twice the molar amount of acetylene. The tetrachloroethane-^{14}C was condensed into a bulb containing phosphorus pentoxide in order to dry it completely and then condensed into a constricted tube cooled in liquid air, and sealed off.

Gas chromatographic analysis of the product from an inactive run showed about 2.5 per cent of impurities, which were separated from tetrachloroethane by gas chromatography with TD and column no. 1 at 108°. The crude tetrachloroethane-^{14}C was injected into the chromatograph with a gastight Hamilton syringe (50 μl) and the fraction corresponding to the tetrachloroethane peak condensed in a freeze-trap at − 80°. The purified product was diluted with 0.48 ml of inactive 1,1,2,2-tetrachloroethane and divided into 7 μl portions in sealed ampoules, which were stored at − 20°. Specific activity 0.51 μci/mg.

Five microlitres of the purified ^{14}C-tetrachloroethane was dissolved in 100 ml of inactive tetrachloroethane. The mixture was fractionally distilled to constant activity, the level of which, for two experiments, was 97–98 per cent of the initial value. The purified product was also assayed for trichloroethylene and tetrachloroethylene by isotope dilution analysis. No activity due to tetrachloroethylene was found and only 0.03 per cent was due to trichloroethylene.

Cis- and trans- 1,2-dichloroethylene. A commercial product consisting of a mixture of the isomers was fractionally distilled. The fractions with a constant b. p. of 60 and 48°, respectively, were tested for purity by gas chromatography with columns nos. 1 and 3. Each fraction contained less than 1 per cent of the isomer.

Dichloroacetaldehyde hydrate. Commercial dichloroacetaldehyde (T. Schuchardt) was distilled and the fraction boiling at 90–92° was immediately used for preparation of the hydrate according to Oddo & Mameli (1903). Repeated crystallization from benzene gave deliquescent crystals of m. p. 42–46°.

Dichloroacetic-1,2-^{14}C acid. Urine collected for 2 days from a mouse dosed with

1,1,2,2-tetrachloroethane-^{14}C (5 µl) was acidified with sulphuric acid and extracted continuously with ether overnight. The ether extract was evaporated to dryness and the acids neutralized with ammonium hydroxide. The ammonium salts were separated by paper chromatography using solvent system no. 1. The zone corresponding to dichloroacetate was extracted with water, the solution evaporated to dryness, and the residue dissolved in isotonic saline (0.6 ml). An assay of dichloroacetic acid by isotope dilution analysis showed that 83 per cent of the activity was due to this compound. Without further purification the solution (3.0 µmol ^{14}C-dichloroacetic acid/ml, calculated from the specific activity of ^{14}C-tetrachloroethane) was used for determining the dichloroacetic acid turnover *in vivo*.

Animals. Female albino mice were used as previously described (YLLNER 1971a).

Methods.

Degradation of tetrachloroethane in neutral aqueous solution. Tetrachloroethane (200 µl) was dissolved in 30 ml of ethanol and 70 ml of a 0.2 M potassium phosphate buffer, pH 7.0. The reaction mixture was divided into 10-ml portions in sealed glass ampoules, which were incubated at 37°. The content of each ampoule was then added to 1 ml of hexane and 50 ml of water, the mixture was shaken, and the aqueous phase discarded. Gas chromatography (TD) was performed using column no. 2 at 50°. The amounts of tetrachloroethane, tri- and tetrachloroethylene were determined by comparison with reference solutions of these compounds in hexane.

Gas chromatography. Gas chromatographic analysis was performed with: (a) A Perkin-Elmer fractometer no. 116 equipped with a thermistor detector (TD); (b) A modification of the same model equipped with an electron capture detector (ECD); (c) A Perkin-Elmer fractometer no. 116 E equipped with a flame ionization detector (FID). Separations were effected with 2 m columns (inner diameter ¼") containing the following stationary phases absorbed on 10 times their weight of Chromosorb W, 60–80 mesh: (1) Octylphthalate, (2) silicone SE 52 and (3) polyethylene glycol 1500. The carrier gases were helium (TD and FID) and nitrogen (ECD).

Dosage and collection of metabolites. Inactive or ^{14}C-labelled tetrachloroethane (10 per cent solutions in olive oil) was injected intraperitoneally by means of a calibrated gas-tight 50 µl Hamilton syringe. The mouse was then placed in an all-glass chamber supplied with a constant slow current of dry air free of carbon dioxide. From the chamber the air was first passed through an ice-cooled U-tube to remove most of the breath-borne moisture and then through two sintered glass thimble traps, the first (trap 1) containing ice-cooled cellosolve (ethylene glycol monoethyl ether) and the second (trap 2) toluene cooled with dry ice in alcohol. The efficiency of these traps was tested with various chlorinated hydrocarbons. Trichloroethylene and less volatile compounds were found to be quantitatively retained, but in the case of *cis*- and *trans*-dichloroethylene about 10 per cent escaped during 24 hours. Finally, the air was passed through a trap (trap 3) containing one part of ethanol amine in two parts of methyl cellosolve (ethylene glycol monomethyl ether) to absorb carbon dioxide. Urine and faeces were collected at the same time as the volatile metabolites, and each collection period was 24 hours. The mouse was fed glucose solution (5 per cent) *ad libitum* but no food. The experiments with labelled tetrachloroethane were usually run for 3 days, and at the end of the third day the mouse was sacrificed by decapitation and homogenized in a Bühler apparatus. The finely ground brei was diluted with water to give a homogeneous suspension (250 ml).

Determination of radioactivity. The radioactivity of the samples was determined with a liquid scintillation spectrometer (Packard Tri-carb, model 3314). Is

were of the low-potassium, tin-foil lined screw-cap type. The freezer was operated at − 1°. The internal standard was ^{14}C-toluene (Packard Instrument Co.). The solvent systems used were:

I. Toluene containing 0.75 per cent of PPO and 0.045 per cent of dimethyl POPOP. Ten millilitres of this solution was mixed with 5 ml of a toluene solution of the sample to be analyzed.

II. One part of xylene, 3 parts of dioxane, and 1 part of cellosolve were mixed. In the solvent mixture 1.4 per cent of PPO, 0.07 per cent of dimethyl POPOP, and 11.2 per cent of naphtalene were dissolved. Ten millilitres of this solution was mixed with 3 ml of cellosolve and 1 ml of an aqueous solution of the sample to be analysed (essentially by the method of BRUNO & CHRISTIAN 1961).

III. In a mixture of 2 parts of toluene and one part of methyl cellosolve 0.55 per cent of PPO was dissolved; 15 ml of this solution was mixed with a 3-ml sample of ethanolamine methyl cellosolve mixture (1 : 2 v/v) containing labelled carbon dioxide (JEFFAY & ALVAREZ 1961).

Quenching determinations were made with all the substances used in the isotope dilution experiments and with the various fractions collected from the animal.

Volatile metabolites.

1. *Examination by gas chromatography.* The contents of traps 1 and 2 containing volatile metabolites (24 hours) from a mouse dosed with 10 µl of tetrachloroethane were combined and the mixture extracted twice with water to remove cellosolve. After drying the toluene layer with anhydrous sodium sulphate it was diluted with toluene to 25 ml. The toluene solution was examined by gas chromatography using the ECD (column no. 3) and FID (column no. 1).

2. *Determination of chlorinated hydrocarbons by isotope dilution.* ^{14}C-tetrachloroethane (5 µl) was injected in a mouse and expired metabolites were collected for 24 hours as described above. The contents of trap 1 (10 ml) were washed out with cellosolve. This solution was added to a mixture of 50 ml each of trichloroethylene (analytical grade, Baker Chemicals), tetrachloroethylene (min. 99 per cent, Merck AG), and tetrachloroethane. This solvent mixture was repeatedly extracted with water to remove cellosolve, and then separated by distillation into 3 fractions containing each of the chlorinated hydrocarbons. Each fraction was then repeatedly distilled to constant activity, which was measured in solvent system no. I.

The contents of trap 2 were washed out with toluene, after which the 3 chlorinated hydrocarbons were added and repeated fractional distillations performed in the same way.

Particular attention was paid to the first few millilitres of the most volatile fraction from each trap. An elevated activity would indicate the presence of a metabolite more volatile than trichloroethylene. In an identical experiment the contents of traps 1 and 2 were combined, *cis*- and *trans*-dichloroethylene (50 ml of each) were added together with tetrachloroethane (50 ml), and the mixture was submitted to fractional distillation. The *cis*- and *trans*-dichloroethylene fractions were redistilled to zero activity.

Urinary metabolites.

1. *Paper chromatography.* ^{14}C-Tetrachloroethane (5 µl) was administered to a mouse by intraperitoneal injection and the urine collected for 2 days. After acidification with sulphuric acid the urine was extracted continuously with ether for 16 hours by a rocking-vessel procedure (YLLNER 1971a) whereby ether-soluble acids were transferred

to 0.1 N sodium hydroxide. The alkaline extract was passed through a cation exchanger (Dowex 50 W X 4) in the H^+ state and the solution of free acids was concentrated *in vacuo* at room temperature. Separations were made on Whatman no. 1 filter paper by the descending technique, using the following solvent systems: (1) Butanol-1.5 N ammonium hydroxide (1 : 1 v/v), (2) butanol-acetic acid-water (4 : 1 : 5 v/v), and (3) ethanol-conc. ammonium hydroxide-water (16 : 1 : 3 v/v). In the case of solvent systems 1 and 3 the extract was made slightly alkaline with ammonium hydroxide prior to spotting on the chromatograms. The distribution of the activity in the radiochromatograms was determined by cutting the 3.5 cm-wide chromatograms into 1-cm strips perpendicular to the direction of migration, and measuring the activity of each strip dipped into a counting vial with solvent system II. Reference strips containing mono-, di- and trichloroacetic acid were sprayed with the $AgNO_3 - NH_4OH$ reagent of MAYER (1957).

2. *Determination by isotope dilution analysis.*

a. *Chloroacetic acid.* Chloroacetic acid (analytical grade, British Drug Houses, Ltd) (500 mg) was added to 2 ml of urine. The solution was neutralized with N sodium hydroxide and made slightly acid with hydrochloric acid. Excess S-benzylthiuronium chloride (1 g) dissolved in a few millilitres of water was added; the benzothiuronium salt of chloroacetic acid crystallized immediately. The product was repeatedly crystallized from dioxane to zero activity. M. p. 159° (decomp.); no depression occurred on mixing with the authentic S-benzylthiuronium salt of chloroacetic acid.

b. *Dichloroacetic acid.* Dichloroacetic acid (min. 98 per cent, British Drug Houses, Ltd.) (500 mg) was added to 2 ml of urine, and the S-benzylthiuronium salt was prepared as in the case of chloroacetic acid. The product was repeatedly crystallized from dioxane to constant activity. M. p. 178° (decomp.); no depression occurred on mixing with the authentic S-benzylthiuronium salt of dichloroacetic acid.

c. *Trichloroacetic acid.* Trichloroacetic acid (analytical grade, Merck AG, Darmstadt) (500 mg) was added to 2 ml of urine and the S-benzylthiuronium salt was prepared as in the case of chloroacetic acid. The product was repeatedly crystallized from methanol-water to constant activity. M. p. 148° (decomp.); no depression occurred on mixing with the authentic S-benzylthiuronium salt of trichloroacetic acid.

d. *2-Chloroethanol.* 2-Chloroethanol (min. 99 per cent, British Drug Houses, Ltd.) (500 mg) was added to 2 ml of urine. The solution was acidified with 1 ml of concentrated hydrochloric acid and kept in a sealed ampoule at 100° for 1 hour. The contents of the ampoule were transferred to a percolator and continuously extracted with ether for 4 hours. The ether solution was dried with anhydrous sodium sulphate and evaporated to a thin syrup, which was diluted with benzene (5 ml). 3,5-Dinitrobenzoyl chloride (1.3 g) was added together with 5 ml of pyridine. After 1 hour at room temperature the reaction mixture was diluted with ether and transferred to a separating funnel. The ether solution was washed first with 5 per cent sodium carbonate solution, then with N sulphuric acid and finally with water. The ether solution was evaporated to dryness, leaving a crystalline mass of the dinitrobenzoate. The product was repeatedly crystallized from benzene-hexane, ethyl acetate-hexane, or ethanol, but in no case did the decreasing activity reach a constant level. However, the final activity corresponded to less than 1 per cent of the dose. M. p. 90°. No depression occurred on mixing with authentic β-chloroethyl-3,5-dinitrobenzoate.

e. *2,2-Dichloroethanol.* 2,2-Dichloroethanol (prepared by the method of SROOG & WOODBURN 1952) (500 mg) was added to 2 ml of urine. Acid treatment and preparation of the dinitrobenzoate were performed as for 2-chloroethanol. Here, too, constant activity was not obtained with repeated crystallization, but the final activity ne-

exceeded 2 per cent of the dose. M. p. 95°; no depression occurred on mixing with authentic β,β-dichloroethyl-3,5-dinitrobenzoate.

f. *2,2,2-Trichloroethanol.* 2,2,2-Trichloroethanol (purified technical product, 98 per cent) (500 mg) was added to 2 ml of urine. Acid treatment and preparation of the dinitrobenzoate were performed as for 2-chloroethanol. Repeated crystallization was performed from ethyl acetate-hexane to constant activity. M. p. 141°; no depression occurred on mixing with authentic β,β,β-trichloroethyl-3,5-dinitrobenzoate.

g. *Glyoxylic acid.* As reported by HOCKADAY *et al.* (1965), glyoxylate reacts at room temperature with a number of substances in normal urine. This can be prevented by acidification, and 0.1 ml of 5 N hydrochloric acid was therefore added to the tube receiving the mouse urine. To a 10-ml specimen of the acidified urine 50 mg of glyoxylic acid was added and the mixture treated with a 0.1 per cent solution of 2,4-dinitrophenyl hydrazine in 2 N hydrochloric acid. The precipitate was filtered off and repeatedly crystallized from aqueous ethanol to constant activity. M. p. 190°; no m. p. depression was found on mixing with authentic glyoxylic acid 2,4-dinitrophenylhydrazon.

h. *Hippuric acid.* Hippuric acid (min. 99 per cent, Merck AG, Darmstadt) (500 mg) was dissolved in a small amount of 2 N sodium hydroxide, and added to 2 ml of urine. Hippuric acid was precipitated from the solution by acidifying with concentrated sulphuric acid. The precipitate was filtered off and repeatedly crystallized from water to constant activity. M. p. 188°; no m. p. depression was found on mixing with authentic hippuric acid.

i. *Oxalic acid.* Oxalic acid (analytical grade, Merck AG, Darmstadt) (500 mg) was dissolved in 10 ml of 10 per cent calcium chloride solution acidified with hydrochloric acid; 2 ml of urine was added and calcium oxalate precipitated by adding 2 N ammonium hydroxide dropwise to the boiling solution. The precipitate was filtered off, washed with water and dried. The activity was determined by oxidation of an aliquot to carbon dioxide as described by JEFFAY & ALVAREZ (1961). Reprecipitation to constant activity was performed by adding ammonium hydroxide to an acidified solution of the calcium oxalate.

j. *Urea.* Urea (analytical grade, Merck AG, Darmstadt) (1 g) was added to 2 ml of urine and the bisxanthyl derivative prepared as described by GESSNER *et al.* (1961). Repeated crystallization was performed from dioxane-water to constant activity. M. p. 272°. No m. p. depression occurred on mixing with the authentic bisxanthyl derivative of urea.

Activity of the faeces and residual activity. The combined faeces, collected for 3 days, were oxidized with a chromic acid – nitric acid mixture (VAN SLYKE & FOLCH 1940) and the labelled carbon dioxide was estimated as described by JEFFAY & ALVAREZ (1961). Aliquots of the suspension of the homogenized mouse were treated similarly and the mean activity was determined from 2 or 3 samples.

Turnover of labelled dichloroacetic acid in vivo. The saline solution of dichloroacetic-1,2-^{14}C acid prepared as described above was injected intraperitoneally into 2 mice (dose 0.6–0.9 µmol, 2.8–4.8 mg/kg). The excretion of activity in the urine and expired air was followed for 72 hours. The dichloroacetic acid in the urine was determined by isotope dilution analysis.

Hippuric acid formation. ^{14}C-Tetrachloroethane (36–52 µmol) and 0.25 ml of a solution of sodium benzoate (156 µmol) were administered simultaneously to a mouse by intraperitoneal injection and the urine was collected for 24 hours. Labelled hippuric acid in the urine was assayed by isotope dilution analysis.

Examination of urine from mice receiving dichloroacetaldehyde. Urine was collected for 24 hours from mice receiving an aqueous solution (0.5 ml) of dichloroacetaldehyde hydrate (8 mg) by intraperitoneal injection. (In a pilot study the LD50 was found to be 10–15 mg). Extraction and paper chromatography (solvent system 1) of ether soluble acids were performed as described above. The urine was also examined for other chlorinated metabolites. It was passed through a cation exchange resin (Dowex 50 W – X 4) in the H^{+} state and neutralized with ammonium hydroxide, after which paper chromatograms were prepared using solvent systems nos. 1 and 3. All the chromatograms were sprayed with the $AgNO_3$–NH_4OH reagent

Results

Non-enzymic degradation of 1,1,2,2-tetrachloroethane at pH 7. Tetrachloroethane is rapidly dehydrochlorinated by alkali with the formation of trichloroethylene. Since this was found in the expired air from mice receiving tetrachloroethane, an examination was made of the breakdown of tetrachloroethane in neutral aqueous solution. Tetrachloroethane was therefore dissolved in a neutral phosphate buffer and the reaction followed by gas chromatographic analysis. Trichloroethylene was formed slowly and in 24 hours 12 per cent of the tetrachloroethane was dehydrochlorinated. In addition, tetrachloroethylene equivalent to 2 per cent of the tetrachloroethane was formed, giving evidence of some oxidation under these conditions.

Excretion of activity and oxidation to carbon dioxide. The distribution of the activity in the expired air, urine and faeces after intraperitoneal injection of ^{14}C-tetrachloroethane is shown in table 1. The elimination of metabolites

Table 1.

Percentage distribution of the activity excreted from mice given 1,1,2,2-tetrachloroethane-1,2-^{14}C

Dose g/kg	0–24 hrs			24–48 hrs			48–72 hrs			Faeces total	Residue	Total
	Traps 1 + 2[a]	Trap 3[b]	Urine	Traps 1 + 2	Trap 3	Urine	Traps 1 + 2	Trap 3	Urine			
0.21	3 4	36 8	27.1	0 1	5.3	2.0	0.0	3.2	0.7	1.3	13.7	93.6
0.23	3 8	51.4	20.5	0 1	5 4	1.0	0 0	4.4	1.1	0 3	14 6	102.6
0.24	3 6	37 0	25 7	0.2	5.9	2.3	0.0	3.6	0 7	0 4	19.1	99.3
0.27	4.1	46 2	24.2	0 3	5.7	1.8	0 0	3.3	0.9	0.7	18.3	105.5
0.32	4.2	36.9	31 5	0.1	5.4	1.4	0.1	2.3	1.2	0.9	11.2	95.2
Means	3.8	41.7	25 8	0 2	5.5	1.7	0 0	3.4	0.9	0 7	15.5	99 2

[a] Traps 1 + 2 contain expired chlorinated hydrocarbons.
[b] Trap 3 contains respiratory carbon dioxide.

was followed for 3 days. Fifty per cent of the dose (range 45–61) was oxidized to carbon dioxide, about 4 per cent was trapped in cellosolve and toluene, and 28 per cent (range 23–34) was excreted as urinary metabolites. After 3 days, 16 per cent (range 11–20) still remained in the animal. Less than one per cent of the dose was found in the faeces, and at least part of this activity was due to contamination with urine.

Chlorinated hydrocarbons in the expired air. Part of the exhaled activity was due to compounds which were trapped in cellosolve and toluene. Examination of these fractions by gas chromatography (ECD) showed peaks corresponding not only to unchanged tetrachloroethane but also to tri- and tetrachloroethylene. The presence of these compounds was confirmed by isotope dilution, fractional distillation, and repeated distillation of each fraction to constant activity.

Trichloroethylene was found mainly in trap 2, and the activity corresponded to 0.2–0.4 per cent of the dose (expired in the first 24 hours from 3 separate animals). The same amount of tetrachloroethylene (0.2–0.4 per cent of the dose) was found to be distributed equally between traps 1 and 2. The remaining activity was due to unchanged tetrachloroethane, which was almost completely trapped in the cellosolve (trap 1).

As the first few millilitres distilled from each of these traps did not show a higher specific activity than the subsequent part of this fraction (trichloroethylene), there was no evidence of any metabolite more volatile than trichloroethylene. Nor did determinations of *cis*- and *trans*-dichloroethylene by isotope dilution analysis in a separate run indicate the presence of any of these compounds. The possibility that this result was due to incomplete trapping of dichloroethylene may be excluded, since under the obtaining conditions about 90 per cent of the respective isomers was found to be trapped. When either *cis*- or *trans*-dichloroethylene was administered by intraperitoneal injection most (90 per cent) of the dose (10 µl) was exhaled unchanged in less than 24 hours.

Urinary metabolites. Radiochromatograms of ether-soluble acids isolated from urine were prepared with 3 solvent systems. In all chromatograms the activity distribution showed peaks corresponding to dichloro- and trichloroacetic acid. There was also other peaks, but these metabolites were not identified.

The results of the urinary metabolite assay by isotope dilution analysis are shown in table 2. Of the identified metabolites dichloroacetic acid was the predominant one, corresponding to about 25 per cent of the urinary activity. Appreciable amounts of trichloroethanol (after hydrolysis) and oxalic acid were also found, together with smaller amounts of trichloroacetic acid, glyoxylic acid and urea. No chloroacetic acid was detected. Small amounts of chloro- and dichloroethanol could not be excluded, but the isotope dilution

Table 2.

Isotope dilution analysis of urinary metabolites from mice receiving 1,1,2,2-tetrachloroethane-^{14}C. Dose 0.16–0.32 g/kg. Percentage of urinary activity excreted in 24 hours.

	% of urinary activity	
	Mean[a]	Range
Dichloroacetic acid	27 (7)	20–34
Trichloroacetic acid	4 (4)	2–8
Trichloroethanol	10 (5)	3–15
Oxalic acid	7 (7)	5–10
Glyoxylic acid	0.9 (4)	0.4–1.4
Urea	2 (2)	2–3

[a] The figures in brackets denote the number of animals examined.

experiments showed that the former did not exceed 1 per cent and the latter, 2 per cent of the dose. Almost half of the urinary activity could not be accounted for.

Dichloroacetic acid turnover in vivo. A considerable part of the urinary activity was due to dichloroacetic acid corresponding on an average to 7 per cent of the dose in 24 hours. To ascertain whether this compound is the stable end product of one metabolic pathway from tetrachloroethane or an intermediary product, which is further metabolized, 2 experiments were designed:

(a) Dichloroacetic acid (10 mg, 78 μmol) as the sodium salt in aqueous solution (0.5 ml) was injected intraperitoneally into a mouse and the urine collected for 24 hours. Ether-soluble acids were extracted and examined by paper chromatography (solvent system 1). Comparison of the spot on the

Table 3.

Percentage distribution of the ^{14}C-activity[a] in 3 days after intraperitoneal injection of dichloroacetic-1,2-^{14}C acid.

Dose ^{14}C-dichloroacetic acid		Percentage of dose			
μmol	mg/kg	Urine	CO_2	Residue	Excreted dichloroacetic acid
0 60	2.8	22	38	24	0 6 (2.5)[b]
0 91	4 8	26	–	–	1 0 (3.8)

[a] The product injected was not pure. Only 83 per cent of the activity was d dichloroacetic acid.

[b] The values in brackets are percentages of urinary activity.

Table 4.

Conversion of ^{14}C-tetrachloroethane into hippuric acid. ^{14}C-Tetrachloroethane and sodium benzoate were injected simultaneously and the urine collected for 24 hours.

Dose ^{14}C-tetrachloroethane g/kg	Dose ^{14}C-tetrachloroethane µmol	Dose sodium benzoate µmol	Percentage of ^{14}C-tetrachloroethane dose: Urine	Percentage of ^{14}C-tetrachloroethane dose: Hippuric acid
0.22	35.8	156	52.3	20.1 (38.4)[a]
0.29	52.1	156	47.5	21.6 (45.6)
0 31	52.1	156	49.0	23.3 (47.5)

[a] The values in brackets are percentages of urinary activity.

chromatogram with spots of standards showed the urinary dichloroacetic acid present to be about 2 per cent of the dose.

(b) A much lower dose, about 0.1 mg of dichloroacetic-1,2-^{14}C acid was injected and the excreted activity followed for 72 hours. As shown in table 3, about one quarter of the activity was found in the urine, one quarter in the animal and two fifths as carbon dioxide. Examination of the urine showed that only about 1 per cent of the dichloroacetic acid was eliminated unchanged.

Turnover of dichloroacetaldehyde in vivo. To examine whether a metabolic pathway for tetrachloroethane might be *via* dichloroacetaldehyde, this compound (as the hydrate) was injected into mice and the urine examined for chlorinated compounds by paper chromatography. Chromatograms were prepared both with urine (passed through a cation exchange resin) and with an extract of ether-soluble urinary acids. Treatment of the chromatograms with the $AgNO_3$-NH_4OH reagent, however, did not reveal any chlorinated compounds apart from traces of dichloroacetic acid. Comparison with spots from standard solutions showed that less than 1 per cent of the dichloroacetaldehyde was excreted as the corresponding acid.

Conversion in vivo of tetrachloroethane into glycine. The formation of glycine was demonstrated by simultaneous injection of ^{14}C-tetrachloroethane and sodium benzoate followed by estimation of ^{14}C-hippuric acid in the urine. The results are shown in table 4. The urinary activity was about 50 per cent of the dose, which is almost twice as much as if the same dose of tetrachloroethane had been injected without the benzoate. The hippuric acid determination showed that at least 20–23 per cent of the ^{14}C-tetrachloroethane was converted to glycine.

Discussion

The metabolism of tetrachloroethane was found to be fairly rapid and only about 4 per cent was expired unchanged. In 24 hours 60–70 per cent of the dose was found to be excreted as various degradation products, mainly carbon dioxide. The results are summarized in fig. 1, which shows the proposed metabolism of 1,1,2,2-tetrachloroethane. The main route seems to involve a stagewise hydrolytic cleavage of carbon-chlorine bonds *via* dichloroacetic acid to glyoxylic acid (a, b, c), the metabolism of which is previously known (l, m, n). A small amount of tetrachloroethane probably undergoes non-enzymic dehydrochlorination to trichloroethylene (d) which gives rise to the well-known metabolites trichloroacetic acid and trichloroethanol (e, f, g). A minute amount of tetrachloroethane is oxidized to tetrachloroethylene (h), which should contribute, though insignificantly, to the formation of trichloroacetic acid and oxalic acid (i, j) (YLLNER 1961).

It was found in this study, that when tetrachloroethane is dissolved in neutral buffer solution, it is slowly decomposed. At 37°, about one tenth of the tetrachloroethane was consumed in 24 hours, and the main product formed was trichloroethylene. Simultaneously, a small amount (2 per cent) was oxidized to tetrachloroethylene (aerobic conditions). The amount of tetrachloroethane consumed was somewhat larger than would be expected from the report of BRAY *et al.* (1952), who studied the liberation of chloride from tetrachloroethane under similar conditions.

These experiments show that the formation of trichloroethylene and tetrachloroethylene *in vivo* may well be accounted for by the effect on tetrachloroethane of the neutral conditions and of the aerobic environment in parts of the body. Quantitatively, however, these reactions can be of only minor importance, since the excreted amount of trichloroethylene and its metabolites trichloroacetic acid and trichloroethanol was less than 5 per cent of the

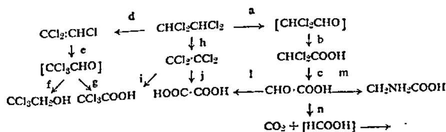


Fig. 1. Suggested metabolic pathways of 1,1,2,2-tetrachloroethane. brackets were not isolated.

amount of tetrachloroethane injected, and less than a half per cent was excreted as tetrachloroethylene. It is evident that 1,1,2,2-tetrachloroethane is primarily metabolized by some reaction other than dehydrochlorination.

In the metabolism of pentachloroethane (YLLNER 1971b) and hexachloroethane (JONDORF 1956; FOWLER 1969), dechlorination, with the formation of trichloroethylene and tetrachloroethylene, respectively, was found to be an important primary reaction. In the case of 1,1,2,2-tetrachloroethane this reaction should yield 1,2-dichloroethylene. However, since even traces of *cis*- or *trans*-dichloroethylene were not found it is unlikely that dechlorination occurs to any appreciable extent.

In the urine of mice treated with tetrachloroethane considerable amounts of dichloroacetic acid were detected. About one quarter of the urinary activity was accounted for as this compound. When labelled dichloroacetic acid was injected intraperitoneally, it was rapidly metabolized and only about one per cent of the dose was excreted unchanged. The excreted activity was largely accounted for by carbon dioxide. Even when greater amounts of dichloroacetic acid (on a molar base, more than twice the dose used with tetrachloroethane) were administered the metabolic breakdown was high and in 24 hours only about 2 per cent was excreted in the urine.

It would appear that, in the metabolism of tetrachloroethane, dichloroacetic acid is an intermediary compound and not a stable end product. The relatively high yield of urinary dichloroacetic acid from tetrachloroethane indicates that the major part of the tetrachloroethane is degraded *via* this metabolite. Even so, the yield of dichloroacetic acid was much higher than would be expected from the experiments performed with injected dichloroacetate. A probable explanation of this discrepancy is that the primary metabolic degradation of tetrachloroethane competitively inhibits the subsequent degradation of dichloroacetate.

The formation of dichloroacetic acid involves the removal of chlorine from one of the carbon atoms in tetrachloroethane. A possible reaction by which this could occur is hydrolytic splitting of the 2 carbon-chlorine bonds with the formation of dichloroacetaldehyde hydrate and the oxidation of this to the corresponding acid (a and b in fig. 1). The fact that when dichloroacetaldehyde hydrate was administered to mice, no chlorinated compounds were detected in the urine apart from traces of dichloroacetic acid indicates that normally dichloroacetaldehyde is rapidly degraded by the removal of chlorine; this is not inconsistent with the metabolism of dichloroacetaldehyde *via* dichloroacetic acid, since the excreted amount of this was low even when it was administered directly.

The metabolism of tetrachloroethane obviously proceeds *via* dichloroacetic acid as an intermediary compound, which then undergoes considerable further metabolization. That this is effected through hydrolytic dehalogenation is

indicated by the small amounts of glyoxylic acid detected in the urine from mice treated with tetrachloroethane.

In their study of the metabolism of ^{14}C-labelled glyoxylic acid in the rat, WEINHOUSE & FRIEDMANN (1951) found that the major part of the activity was excreted as carbon dioxide, oxalic acid and glycine (isolated as hippuric acid). Similar results were reported by SMITH *et al.* (1964) with regard to glyoxylate metabolism in human subjects. NAKADA & WEINHOUSE (1953) found glyoxylate to be oxidized by xanthine dehydrogenase to oxalate and to formate and carbon dioxide by a rat liver enzyme. This glyoxylate dehydrogenase was prepared from the mitochondrial fraction and studied by NAKADA & SUND (1958). The amination of glyoxylate to glycine is catalyzed by glutamic-glyoxylic acid transaminase. *In vitro* studies on the metabolism of 1-^{14}C-glyoxylate by liver mitochondria of the rat and man have shown that decarboxylation, oxidation to oxalate and amination to glycine account for virtually all the glyoxylate metabolized (CRAWHALL & WATTS 1962). Oxalate does not seem to undergo any considerable change *in vivo* (WEINHOUSE & FRIEDMANN 1951), and is an end product of one of the metabolic pathways of glyoxylic acid. Formate is almost entirely oxidized to carbon dioxide by the rat (WEINHOUSE & FRIEDMANN 1951).

From mice receiving labelled tetrachloroethane the above mentioned metabolites of glyoxylate were found to account for the bulk of the excreted activity. Carbon dioxide was equivalent to half the dose and oxalic acid to 2 per cent of the dose. When benzoate was administered, the urinary activity was considerably increased and about 20 per cent of the dose was excreted as hippuric acid.

Acknowledgments

The author is indebted to Dr. Brian Pringe for carrying out the synthesis of labelled tetrachloroethane and to Miss Inga Jakobson for skilful technical assistance.

REFERENCES

Barrett, H. M., J. G. Cunningham & J. H. Johnston: A study of the fate in the organism of some chlorinated hydrocarbons. *J. Ind. Hyg. Toxicol.* 1939, 21, 479–490

Bray, H. G., W. V. Thorpe & D. K. Vallance: The liberation of chloride ions from organic chloro compounds by tissue extracts. *Biochem. J.* 1952, 51, 193–201.

Bruno, G. A. & J. E. Christian: Determination of carbon-14 in aqueous bicarbonate solutions by liquid scintillation counting techniques. *Anal. Chem.* 1961, 33, 1216–1218.

Crawhall, J. C. & W. E. Watts: The metabolism of glyoxylate by human- and rat mitochondria. *Biochem. J.* 1962, 85, 163–171.

Fowler, J. S. L.: Some hepatotoxic actions of hexachloroethane and its sheep. *Brit. J. Pharmacol.* [illegible] 530–542.

Casq, M.: Etudes toxicologique et hygienique des solvents chlorés acethyleniques. Delmas, Bordeaux 1936.

Gessner, P. K., D. V. Parke & R. T. Williams: Studies in detoxication. 86. The metabolism of ^{14}C-labelled ethylene glycol. *Biochem. J.* 1961, 79, 482–489.

Hockaday, T. D. R., E. W. Frederick, J. E. Clayton & L. H. Smith, Jr.: *Studies on* primary hyperoxaluria. II. Urinary oxalate, glycolate, and glyoxylate *measurement* by isotope dilution methods. *J. Lab. Clin. Med.* 1965, 65, 677–687.

Jeffay, H. & J. Alvarez: Liquid scintillation counting of carbon-14. Use of ethanolamine-ethylene glycol monomethyl ether-toluene. *Anal. Chem.* 1961, 33, 612–615.

Jondorf, W. R.: The metabolism of certain chlorinated hydrocarbons. Dissertation, University of London, 1956.

Lehmann, K. B. & Hasegawa: Studien über die Absorption chlorierter Kohlenwasserstoffe aus der Luft durch Tier und Mensch. *Arch. Hyg.* 1910, 72, 327–342.

Lilliman, B.: Suggested mechanism of poisoning by liquid tetrachloroethane. *Analyst* 1949, 74, 510–511.

Mayer, F.: Zum Verhalten von Trichloracetat und anderen Halogenacetaten gegenüber Sulfhydryl- und Aminogruppen sowie einigen sekundären Pflanzenstoffen. *Biochem. Z.* 1957, 328, 433–442.

McKinney, R. M. & G. W. Pearce: Isotope-labeled insecticides. Synthesis of carbon-14-labeled aldrin and dieldrin. *Agricult. Food Chem.* 1960, 8, 456–459.

Nakada, H. I. & L. P. Sund: Glyoxylic acid oxidation by rat liver. *J. Biol. Chem.* 1958, 233, 8–13.

Nakada, H. I. & S. Weinhouse: Studies of glycine oxidation in rat *tissues. Arch. Biochem. Biophys.* 1953, 42, 257–270.

Oddo, G. & E. Mameli: *Preparazione sintetica dell'idrato di bicloraldeide. Gazzetta Chim. Ital.* 1903, 33, 398.

Smith, Jr., L. H., T. D. R. Hockaday, M. L. Efron & J. E. Clayton: The metabolic defect of primary hyperoxaluria. *Trans. Ass. Am. Physicians* 1964, 77, 317–325.

Sroog, C. E. & H. M. Woodburn: 2,2-Dichloroethanol. *Org. Synthesis* 1952, 32, 46–48.

Van Slyke, D. D. & J. Folch: Manometric carbon determination. *J. Biol. Chem.* 1940, 136, 509–541.

Weinhouse, S. & B. Friedmann: Metabolism of labelled 2-carbon acids in the intact rat. *J. Biol. Chem.* 1951, 191, 707–717.

Yllner, S.: Urinary metabolites of ^{14}C-tetrachloroethylene in mice. *Nature* 1961, 191, 820.

Yllner, S.: The metabolism of symmetric tetrachloroethane in mice. *Proc. XV. Int. Congr. Occupat. Health.* Vienna, 1966, 395–399.

Yllner, S.: Metabolism of 1,1,1,2-tetrachloroethane in the mouse. *Acta pharmacol. et toxicol.* 1971a, 29, 471–480.

Yllner, S.: Metabolism of pentachloroethane in the mouse. *Acta pharmacol. et toxicol.* 1971b, 29, 481–489.

Acta pharmacol. et toxicol. 1971, 29, 513–524.

From the National Institute of Occupational Health,
Stockholm, Sweden

Metabolism of ^{14}C-Pentachlorophenol in the Mouse

By

Inga Jakobson and Sven Yllner

(Received July 28, 1970)

Abstract: After subcutaneous or intraperitoneal injection of ^{14}C-pentachlorophenol (^{14}C-PCP) in the mouse (specific activity 1.6 μci/mg, dose 15–37 mg/kg body weight) the distribution of the activity in the body was determined by whole-body autoradiography and by analysis of the individual organs after oxidation to $^{14}CO_2$. The highest specific activity was found in the gall bladder and its contents, the wall of the stomach fundus, the contents of the gastro-intestinal tract and the liver. This shows that there is both a gastric and a biliary secretion of PCP and/or its metabolites, and excretion in the faeces. Most of the activity (72–83 %) was excreted in the urine in four days, about half of the dose in 24 hours. Only traces (< 0.05 %) were detected in the expired air. Identification and estimation of metabolites were performed by paper chromatography and isotope dilution techniques. All the urinary activity in the first 24 hours was due to PCP and probably tetrachlorohydroquinone. At least PCP was excreted in both the free and the conjugated form.

Key-words: Pentachlorophenol – metabolism.

Pentachlorophenol (PCP) and its salts, especially the sodium salt, are widely used as biocides. Owing to the high toxicity of these compounds there have been frequent cases of poisoning. A detailed account of their physical, chemical and toxicologic properties and analytical procedures has been published by BEVENUE & BECKMAN (1967).

With regard to the metabolism of PCP little research seems to have been done. It has long been known that the compound is excreted in the urine, and DEICHMANN *et al.* (1942) suggested that the concentration of PCP in human urine might be used as a measure of exposure. Their experi the rabbit showed that after oral administration most of the PCP changed in the urine. After intraperitoneal injection, however, PCP is excreted unchanged, according to their experiments detoxication through · ion to sulphuric or glucu

occur. Nor were any other metabolites detected. Essentially similar results were obtained in the rabbit by BETTS *et al.* (1955), who found no excretion of PCP in the conjugated form.

Materials and Methods

Melting points are uncorrected.

^{14}C-Pentachlorophenol. The ^{14}C-PCP was synthetized by chlorination of ^{14}C-phenol (JAKOBSON & YLLNER 1969). Sublimation gave a product 97 per cent radiochemically pure, with a specific activity of 1.6 μci/mg.

Animals and dose. The experiments were performed on female mice (NMRI) weighing 20–30 g. A 1.25 per cent solution of ^{14}C-PCP in olive oil was injected with a calibrated Hamilton syringe either subcutaneously or intraperitoneally in doses of 0.25–1 mg.

Collection of metabolites. After the ^{14}C-PCP had been injected the mouse was placed in a glass chamber with a constant slow current of dry carbon-dioxide-free air. The air was conducted from the chamber through an ice-cooled U-tube to remove the greater part of the expired moisture. It was then passed through 2 absorption vessels, the first containing ice-cooled cellosolve (ethylene glycol monoethyl ether) and the other toluene cooled with dry ice in ethanol. Finally the air was passed through a sintered glass thimble trap containing one part of ethanolamine in 2 parts of methyl cellosolve (ethylene glycol monomethyl ether) to absorb carbon dioxide. As the first runs showed that less than 0.05 per cent of the activity was excreted as volatile metabolites the absorption vessels were dispensed with in subsequent work.

The urine and faeces were collected in separate vessels for 24-hour periods. The experiment was run for 4 days, during which time the mouse was given only 5 per cent glucose solution *ad libitum*. Those allowed to live longer than 4 days after this period were given normal food and water. The animals were sacrificed by means of nitrogen. The heart, lungs, liver, kidneys, brain, stomach, intestines and gall bladder were removed and homogenized separately in water by means of an *Ultra-Turrax* apparatus. The remainder of the animal was homogenized to a fine brei with a Bühler unit. The brei was suspended in 250 ml of water and thoroughly mixed. Oxidation to carbon dioxide was performed with 1 ml samples by the method of VAN SLYKE & FOLCH (1940) and the activities were assayed as described by JEFFAY & ALVAREZ (1961). The faeces and organ homogenates were oxidized in the same way. However, when tested with ^{14}C-PCP the oxidation was incomplete. The yield of labelled carbon dioxide was only 70 per cent. The activities obtained for the faeces and tissue homogenates would therefore appear to be on the low side.

Radioactivity determination. Assays were performed on all the specimens with a liquid scintillation spectrometer (Packard Tri-Carb, Model 3314) at − 1°. The internal standard used was ^{14}C-toluene (Packard Instrument Co.).

The determinations were performed with 3 solvent systems:

I. Toluene containing 0.75 per cent of PPO and 0.045 per cent of dimethyl POPOP. Of this solution 10 ml was mixed with 5 ml of a toluene solution of the sample.

II. One part of xylene, 3 parts of dioxane and 1 part of cellosolve were mixed. In

this mixture 1.4 per cent of PPO, 0.07 per cent of dimethyl POPOP and 11.2 per cent of naphthalene were dissolved. Of this solution 10 ml was mixed with 3 ml of cellosolve and 1 ml of the aqueous solution, including urine, from the sample to be analysed (essentially by the method of BRUNO & CHRISTIAN (1961)).

III. Two parts of toluene and 1 part of methyl cellosolve were mixed and 0.55 per cent of PPO was dissolved in the mixture. Of this solution 15 ml was mixed with 3 ml of an ethanolamine-methyl cellosolve solution (1 : 2 v/v) containing labelled carbon dioxide (JEFFAY & ALVAREZ 1961).

Autoradiography. Three mice were injected with 1 mg of ^{14}C-PCP subcutaneously and sacrificed with ether after 4, 20 and 100 hours. The animals were then rapidly cooled to −78° with dry ice in hexane, during which process not more than about 2 per cent of the activity was leached out. After drying at −15° for 24 hours they were embedded in semiliquid carboxymethyl cellulose gel and rapidly frozen to blocks. Sectioning was performed at −15°. A strip of adhesive tape was affixed to the surface of the block before sectioning so that unbroken sections of the whole animal adhering to the tape were obtained. These were freeze-dried and placed on the photographic film (Kodirex and Strukturix) After the exposure the sections and films were separated and the films developed (ULLBERG 1954, 1958, 1969).

Isotope dilution analysis.

Pentachlorophenol. To an aliquot of urine (2–5 ml) 300 mg of carrier pentachlorophenol (Fluka AG, puriss) was added, followed by enough sodium hydroxide to dissolve it. On acidification PCP was precipitated and filtered off. It was repeatedly crystallized from *iso*-octane to a constant activity (m.p. 190°). The samples were analysed by dissolution in scintillator solution II (without water). In the determination of conjugated PCP the urine was treated with 3 N hydrochloric acid at 100° for 1 hour in an ampoule. Owing to the low solubility of PCP in acidic solution it was necessary first to hydrolyse the urine before the carrier could be added. The assay was then performed in the same way as for free PCP.

Tetrachlorohydroquinone. In an aliquot of acid treated urine (see above) 400 mg of tetrachlorohydroquinone (TCH) (Eastman, analytical grade) was dissolved with the addition of ethanol. After precipitation with water the crystals were filtered off, dried and repeatedly crystallized from toluene to constant activity (m.p. 240°). The activity was determined in scintillator solution I (10 ml) to which 4 ml of cellosolve had been added.

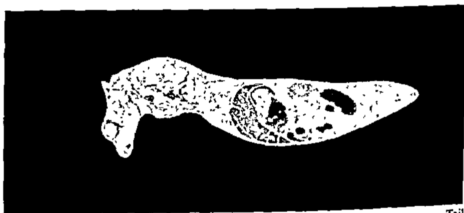
Oxalic acid. In 3 ml of urine and 10 ml of 5 per cent hydrochloric acid 500 mg of oxalic acid (Merck AG, analytical grade) was dissolved. Five ml of 10 per cent calcium chloride was added and calcium oxalate was precipitated by adding 2 N ammonium hydroxide to the boiling solution; it was then filtered, washed with water and dried. It was repeatedly crystallized from 5 per cent hydrochloric acid and 2 N ammonium hydroxide until the activity was zero. To determine the activity the oxalate was oxidized to carbon dioxide in the same way as the homogenized mouse.

Paper chromatography. Paper chromatographic analysis of the urine was performed on Whatman paper no. 1 by the descending method. The solvent mixtures were *iso*-amyl alcohol : ammonium hydroxide : water (6 : 3 : 1 v/v/v) (IV) (SITOFL & SCHL 1953) and butanol-1 : acetic acid : water (4 : 1 : 5 v/v/v) (V). The activity was by analysing 1 cm strips separately in scintillator solution II (without additi solve). As, in preliminary experiments, a large portion of the activity

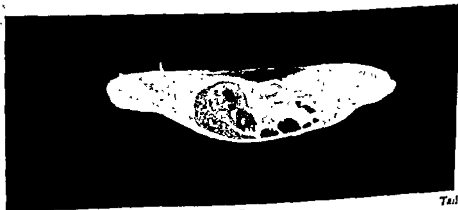
evaporation of the urine in neutral and acidic solution, ammonium hydroxide was added to the urine before evaporation, after which the concentrated solution was placed on the paper chromatogram. Hydrolysable compounds were examined by treating the urine with 3 N hydrochloric acid at 100° for 1 hour in ampoules. The hydrolysate was examined in 2 ways: (1) The solution was evaporated directly to dryness (which resulted in a considerable loss of PCP) and the remainder was spotted on the chromatogram. (2) The hydrolysate was extracted with ether; to the ethereal solution ammonium hydroxide was added and the liquid evaporated to dryness for preparation of chromatograms. (The acid extraction residue was also evaporated to dryness and chromatographed). The PCP and TCH references were detected by spraying the chromatograms with a fresh 1 : 1 mixture of 15 per cent ferric chloride and 1 per cent potassium ferricyanide solutions (Pospíšil & Taimr 1965).

Results

The excretion of the activity in the urine and faeces and the distribution and specific activities in the various organs after intraperitoneal injection of



Head A Tail



Head B Tail

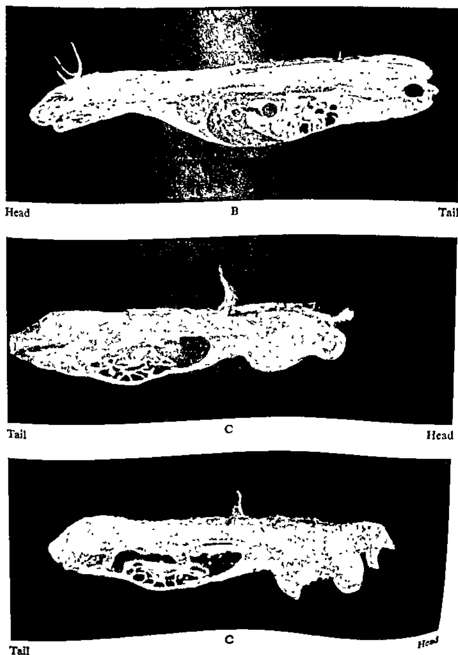


Fig. 1. Whole-body autoradiogram of mice after subcutaneous injection of ^{14}C-pentachlorophenol: after 4 hours (A), 20 hours (B), and 100 hours (C)

Table 2.

Labelled compounds in the urine collected 0–24 hours after intraperitoneal injection of ^{14}C-pentachlorophenol in the mouse. Percentage of injected dose. In brackets percentage of urinary activity.

Dose		^{14}C-PCP in urine									^{14}C-TCH in urine			Recovered activity
		Before acid treatment			After acid treatment			Difference			After acid treatment			
mg	mg/kg	% dose	% ur. ac.	Mean			Mean			Mean			Mean	
1	35.5	29[a]	(48)[a]		49[a]	(82)[a]		20[a]	(34)[a]					
1	36.8	32	(54)	29 (50)	41	(70)	38 (66)	9	(16)	9 (16)				
1[b]	35.7	27	(48)		30	(53)		3	(5)					
1	37.2	27	(49)		32	(58)		5	(9)		26	(48)		(106)
0.5	18 6	35	(53)		39	(59)		4	(6)					
0.5	18.2	24	(52)	30 (54)	29	(63)	36 (66)	5	(11)	7 (12)				
0.5	16.6	36	(58)		48	(77)		12	(19)					
0.5	14.8	24	(54)		29	(66)		5	(12)		15	(33)		(99)
0.25	8.1				26	(56)					20	(43)		(99)
0.25	7.4				26	(54)	26 (54)				21	(44)	21 (44)	(98)
0 25	8.2				26	(53)					21	(44)		(97)

[a] Means of 2 analyses.
[b] Subcutaneous injection.

the activity was chiefly distributed between 2 peaks with Rf values of 0.6 and 0. The former peak was eluted with water and renewed chromatography of the evaporated eluate with solvent IV then gave a peak consistent with the Rf value of 0.8 for PCP (0.78 according to SIEGEL & SCHLÖGL (1953)). ^{14}C-PCP was assayed by isotope dilution; the whole activity of the peak proved to come from this compound.

The activity at the start of the chromatogram was eluted in the same way and the eluate was treated with acid. PCP and TCH analyses by isotope dilution then showed these 2 compounds to be present in equal proportions. Evaporation of the acidic solution (when part of the activity disappeared) and renewed chromatography with solvent IV gave 2 peaks corresponding to TCH (Rf = 0) and PCP. By direct hydrolysis of the urine followed by evaporation, ether extraction and chromatography in solvent IV, the activity was also obtained chiefly at the start and at the position for PCP.

Chromatography of the urine in solvent V showed that the main activity followed the front like TCH and PCP. Elution of this region and isotope dilution analysis showed that about 63 per cent of the activity of the peak derived from PCP, which corresponds to 53 per cent of the urinary activity. This is the same value as was obtained by direct isotope dilution analysis of the urine. On all chromatograms the amounts of PCP and TCH were too small to be detected by the colour reagent.

Discussion

Intraperitoneal or subcutaneous injection of ^{14}C-pentachlorophenol in the mouse was followed by rapid absorption and excretion of the activity. About one third of the amount injected was excreted in the form of unchanged PCP in the urine. As well as free PCP, some PCP was apparently found in the conjugated form. No carbon dioxide was formed and tetrachlorohydroquinone was the only breakdown product detected. Together with the PCP after hydrolysis it accounted for the whole of the urinary activity excreted in the first 24 hours. The extent to which TCH occurred in the conjugated form could not be established with certainty.

With regard to the sodium pentachlorophenolate injected intraperitoneally in the rat it was found by DEICHMANN *et al.* (1942) that of a dose corresponding to 2.4 mg of PCP 12.5 per cent was excreted during the first 24 hours while 47.0 per cent remained in the animal. Thus, during this period 40.5 per cent had been converted to other products, which, however, were not identified. In one animal 13 per cent of the PCP remained after 6 days. After oral administration of the sodium salt in amounts corresponding to 37 mg of PCP per kg in the rabbit, about 70 per cent was excreted in tl

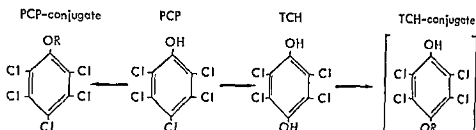


Fig. 2. Suggested metabolic fate of PCP.

hours but only 0.3 per cent in the faeces. The largest amounts were located in the stomach and intestines, the muscles and the blood (5, 5 and 4 per cent, respectively), while smaller amounts were found in other organs – for instance, 1–2 per cent in the liver and gall bladder, together. It was also found that in the rabbit only traces of excreted PCP were present as conjugates with sulphuric or glucuronic acid, and BETTS *et al.* (1955) found no excretion of conjugated PCP.

With regard to the rate of excretion and the distribution between the organs the results of the present study in the mouse are thus consistent with those reported by DEICHMANN *et al.* (1942) for other species. Also in respect of the amount of converted PCP our results are in agreement with the calculations of DEICHMANN *et al.* (1942) on the difference between the dose and the recovered PCP in the rat. In the rabbit, on the other hand, the breakdown during the first 24 hours was only 9 per cent. It should be noted, however,

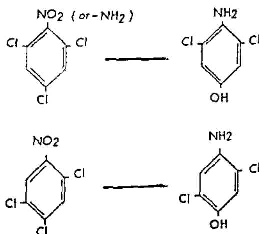


Fig. 3. Hydrolytic dehalogenation of some aromatic compounds.

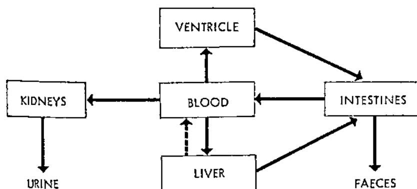


Fig. 4. The turnover of PCP and/or its metabolites in the mouse.

that these investigators used an analytical method (colour reaction with nitric acid after steam distillation of acidified samples) by which the same results would be obtained for TCH as PCP.

In contrast to the findings in the rat and the rabbit, the results of the present study in the mouse show that part of the PCP was excreted in a conjugated form. The nature of this conjugate was not investigated.

Hydrolytic dechlorination of aromatic compounds, the process by which TCH is formed *in vivo* from PCP (fig. 2), has been observed in the rabbit with 2,4,6-trichloronitrobenzene and -aniline and possibly with 2,4,5-trichloronitrobenzene in paper chromatographic examination of urine metabolites (fig. 3) (BETTS *et al.* 1957). Hydrolytic dechlorination is, however, only a subsidiary reaction in the metabolism of these compounds.

A number of organic compounds, most of them basic, are secreted by gastric juice, a process that was almost ignored until SHORE *et al.* (1957) published a hypothetical explanation of this process. The recent literature has been reviewed by HART *et al.* (1969). The high specific activity in the wall of the stomach fundus and the stomach contents evident in the autoradiograms (fig. 1) shows that PCP is also secreted in this way. The activity in other parts of the wall of the gastrointestinal tract affords no evidence of any secretion there. The high specific activity of the gall bladder and its contents, however, shows that biliary secretion is also important in the metabolism of PCP. The excretion of PCP and its metabolites thus occurs via the kidneys and by the processes of gastric and biliary secretion; the greater part being passed in the urine. The detoxication occurs to a considerable extent by direct renal filtration, since about one half the urine activity could be ascribed to non-conjugated PCP. The turnover of PCP and/or its metabolites in the mouse is shown in fig. 4.

Acknowledgement

We wish to thank Dr. Gunnar Nordberg, National Institute of Public Health, for his advice on the autoradiographic preparations.

REFERENCES

Betts, J. J., H. G. Bray, S. P. James & W. V. Thorpe: The metabolism of the trichloronitrobenzenes in the rabbit. *Biochem. J.* 1957, 66, 610–621.

Betts, J. J., S. P. James & W. V. Thorpe: The metabolism of pentachloronitrobenzene and 2, 3, 4, 6-tetrachloronitrobenzene and the formation of mercapturic acids in the rabbit. *Biochem. J.* 1955, 61, 611–617.

Bevenue, A. & H. Beckman: Pentachlorophenol: A discussion of its properties and its occurrence as a residue in human and animal tissues. *Residue Reviews* 1967, 19, 83–134.

Bruno, G. & J. Christian: Determination of carbon-14 in aqueous bicarbonate solutions by liquid scintillation counting techniques. *Anal. Chem.* 1961, 33, 1216–1218.

Deichmann, W., W. Machle, K. V. Kitzmiller & G. Thomas: Acute and chronic effects of pentachlorophenol and sodium pentachlorophenate upon experimental animals. *J. Pharmacol. Exp. Ther.* 1942, 76, 104–117.

Hart, L. G., A. M. Guarino & L. S. Schanker: Gastric dialysis as a possible antidotal procedure for removal of absorbed drugs. *J. Lab. clin. Med.* 1969, 73, 853–860.

Jakobson, I. & S. Yllner: Synthesis of ^{14}C-pentachlorophenol. *Acta Chem. Scand.* 1969, 23, 319–320.

Jeffay, H. & J. Alvarez: Liquid scintillation counting of carbon-14. Use of ethanolamine-ethylene glycol monomethyl ether – toluene. *Anal. Chem.* 1961, 33, 612–615.

Pospíšil, J. & L. Taimr: Antioxydantien und Stabilisatoren VI. Chromatographisches Verhalten der Substanzen vom Typus des Brenzcatechins. *Coll. Czech. Chem. Commun.* 1965, 30, 1513–1522.

Shore, P. A., B. B. Brodie & C. A. M. Hogben: The gastric secretion of drugs. A pH partition hypothesis. *J. Pharmacol. Exp. Ther.* 1957, 119, 361–369.

Siegel, A. & K. Schlögl: Über die Papierchromatographische Trennung von Chlorphenolen und Chlorphenoxyessigsäuren. *Monatshefte für Chemie* 1953, 84, 686–691.

Ullberg, S.: Studies on the distribution and fate of ^{35}S-labelled benzylpenicillin in the body. *Acta Radiol.* 1954, Suppl. 118.

Ullberg, S.: Autoradiographic studies on the distribution of labelled drugs in the body. *Second U. N. Int. Conf. Peaceful Uses of Atomic Energy.* 1958, 24, 248–254.

Ullberg, S.: Vart tar farmaka vägen i kroppen? Distributionsundersökningar med märkta substanser *Läkartidningen* 1969, 66, 47–58.

Van Slyke, D. D. & J. Folch: Manometric carbon determination. *J. Biol. Chem.* 1940, 136, 509–541.

Acta pharmacol. et toxicol. 1971, 29, 525–532.

From the Psychiatric Research Centre, S:t Jörgen Hospital and the Department of Pharmacology, University of Göteborg, Göteborg, Sweden

Changes in the Formation of ^{3}H-Catecholamines from ^{3}H-DOPA and ^{3}H-Tyrosine Induced by Unlabelled DOPA

By

Torgny Persson and Bertil Waldeck

(Received September 24, 1970)

Abstract: Various doses of L-DOPA were given intravenously to mice together with ^{3}H-L-DOPA or ^{3}H-tyrosine. Two hours later the animals were killed. The ^{3}H-noradrenaline (NA) and ^{3}H-dopamine (DA) in the caudate nucleus, the remainder of the brain and the heart were determined. In some experiments only unlabelled L-DOPA was given and NA and DA were determined fluorimetrically. With doses of DOPA 10 and 100 mg/kg, the ^{3}H-NA but not the ^{3}H-DA formed from ^{3}H-DOPA in the brain decreased, indicating a saturation of the DA β-hydroxylase. In the heart a similar decrease was observed with a ten times lower dose. In the caudate nucleus an increased synthesis of ^{3}H-DA from ^{3}H-DOPA was observed following the administration of 100 mg/kg DOPA. The formation of ^{3}H-NA both from ^{3}H-tyrosine and from ^{3}H-DOPA was influenced in a similar way by DOPA. There were no marked changes in the levels of unlabelled amines under the present experimental conditions.

Key-words: Mouse brain – DOPA – catecholamine synthesis.

After the administration of ^{3}H-DOPA in μg quantities to mice, the ratio of ^{3}H-dopamine (DA) to ^{3}H-noradrenaline (NA) in the brain was lower than the "physiologically" normal value (PERSSON & WALDECK 1968). When, on the other hand, ^{3}H-tyrosine was given in the same low dose a "physiological" ratio between the tritiated catecholamines was obtained (SVENSSON & WALDECK 1969). Similar results have been obtained in rats (PERSSON 1969). BARTHOLINI & PLETSCHER (1968) found that the ratio ^{14}C-DA/^{14}C-NA after ^{14}C-DOPA was higher when a high rather than a low dose of the precursor was given. After a large dose of unlabelled DOPA excessive amounts of DA accumulate in the brain, preferentially in the caudate nucleus (BERTLER & ROSENGREN 1959).

In this study the relationship between the dose of ^{3}H-DOPA and the yield of ^{3}H-NA and ^{3}H-DA has been further investigated, particularly with to the formation of ^{3}H-DA in the caudate nucleus. The effect of the formation of ^{3}H- DA from ^{3}H-tyrosine has also b

Material and Methods

Female mice weighing about 20 g were used in all experiments. L-DOPA-2,5,6-^{3}H and L-tyrosine-3,5-^{3}H with specific activities of about 30 ci/mmol, were obtained from The Radiochemical Centre, Amersham. The radiochemical purity was regularly checked by radiopaper chromatography (PERSSON & WALDECK 1970).

To obtain an injectable solution of DOPA at higher doses, it is necessary to dissolve the compound in hydrochloric acid and then adjust the pH to about 5 by e. g. sodium bicarbonate. Due to a deplorable confusion, calcium carbonate was used for this purpose in the experiments presented in tables 1–3. This resulted in Ca^{++} doses which could have been up to about 30 mg/kg. However, table 1 in which calcium carbonate was used and fig. 1 where it was not, contain data from similar experiments. There were no obvious differences in the yield of ^{3}H-NA and ^{3}H-DA between these experimental series. Moreover, in a pilot study the experiments shown in table 1 and 2 with 100 mg/kg of DOPA were repeated with and without Ca^{++}. Again no conclusive effect of Ca^{++} was obtained (data not shown).

The experiments were terminated by decapitation of the animals, after which the brain and the heart were removed for analysis. In some experiments the caudate nucleus was separated from the remainder of the brain after the lateral ventricles had been exposed by sectioning the corpus callosum and the fornix. The tissues were extracted in perchloric acid (BERTLER *et al.* 1958). ^{3}H-NA and ^{3}H-DA were isolated from the extracts by a combination of alumina and DOWEX columns before liquid scintillation counting (PERSSON & WALDECK 1968). Endogenous NA was determined fluorimetrically as described by BERTLER *et al.* (1958)) and DA according to CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962).

Results

Effect of DOPA on the formation of ^{3}H-NA and ^{3}H-DA from ^{3}H-DOPA in the brain and heart.

^{3}H-L-DOPA, 10 µg/kg, was mixed with various amounts of cold L-DOPA and given to mice intravenously. Two hours after the injection the animals were killed. The ^{3}H-NA and ^{3}H-DA in the brain and heart were determined (fig. 1). In doses up to 10 mg/kg of DOPA, the yield of ^{3}H-NA in the brain was not significantly changed. From 10–100 mg/kg, however, the yield was reduced about 3 times ($P < 0.001$). At the lowest DOPA dose, ^{3}H-DA was about one third of the corresponding ^{3}H-NA value. After 100 mg/kg of DOPA, ^{3}H-DA did not show any changes, which indicates that the two ^{3}H-amine levels were equal.

In the heart the yield of ^{3}H-NA at low doses of DOPA was about 6 times higher than in the brain. With 1–10 mg/kg of DOPA this yield was reduced about 3 times ($P < 0.005$). With 100 mg/kg the ^{3}H-NA level showed a tenfold decrease as compared with the value at 1 mg/kg ($P < 0.001$). Thus the difference in yield of ^{3}H-NA between the brain and heart was almost eliminated at the high dose level. As in the brain, the yield of ^{3}H-DA in the heart was not influenced by the dose given. With 100 mg/kg of DOPA, the concentration of ^{3}H-DA was about half that of ^{3}H-NA.

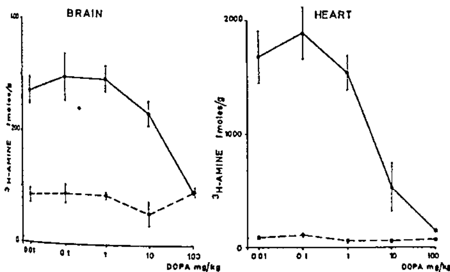


Fig. 1. ³H-noradrenaline and ³H-dopamine in the brain and heart after ³H-DOPA and different doses of unlabelled DOPA. Mice received 10 µg/kg ³H-DOPA together with unlabelled DOPA intravenously. Two hours later the animals were killed. The ³H-catecholamines in the brain and heart were determined. The mean ± S. E. M. of 3 experimental groups each comprising 6 animals are shown. 1 fmol = 10^{-15} mol. Solid line: ³H-noradrenaline. Broken line: ³H-dopamine.

Effect of DOPA on the distribution of ³H-NA and ³H-DA formed from ³H-DOPA in the brain.

This experiment differed from the previous one in that L-DOPA was given in doses of 1 and 100 mg/kg only; moreover the caudate nucleus was dis-

Table 1.

³H-noradrenaline (³H-NA) and ³H-dopamine (³H-DA) in the brain and heart after ³H-DOPA and different doses of unlabelled DOPA. Mice received 10 µg/kg ³H-DOPA together with unlabelled DOPA intravenously. Two hours later the animals were killed. The ³H-NA and ³H-DA in the caudate nucleus, the remainder of the brain and the heart were determined. The mean ± S. E. M. of 6 experimental groups, each comprising 6 animals are shown. The data are expressed in fmoles (10^{-15} mol) per gram tissue.

Unlabelled DOPA mg/kg	Caudate nucleus		Remainder of brain		Heart	
	³H-NA	³H-DA	³H-NA	³H-DA	³H-NA	³H-DA
1	93 ± 8	629 ± 53	266 ± 13	61 ± 3	1561 ± 111	106 ± 14
100	78 ± 16	1204 ± 46	108 ± 5	88 ± 4	190 ± 15	157 ± 11

sected out from the remainder of the brain and analyzed separately. The results are shown in table 1. The level of ^{3}H-DA in the caudate nucleus increased twofold when the dose of DOPA was increased from 1 to 100 mg/kg ($P < 0.001$). ^{3}H-NA was 7 times lower than ^{3}H-DA after the low dose of DOPA and may have further decreased after the high dose. (The estimation of low levels of ^{3}H-NA is subject to a larger error in the presence of relatively large amounts of ^{3}H-DA). In the remainder of the brain the increase in ^{3}H-DA was less i. e. 40–50 per cent ($P < 0.001$). ^{3}H-NA in this part of the brain decreased 2–3 times with increasing doses of DOPA i. e. from 1 to 100 mg/kg ($P < 0.001$).

Since this experiment was carried out four months after the previous one, the heart was analyzed for comparative purposes. The data on the heart from the two experimental series were in good agreement (compare table 1 with fig. 1).

Effect of DOPA on the formation and distribution of ^{3}H-NA and ^{3}H-DA formed from ^{3}H-tyrosine in the brain and heart.

Mice were given ^{3}H-tyrosine, 10 µg/kg intravenously, either alone or together with 10 or 100 mg/kg of L-DOPA. One hour later the animals were killed. The ^{3}H-NA and ^{3}H-DA in the caudate nucleus, in the remainder of the brain and in the heart were determined. The results are shown in table 2. The yield of ^{3}H-DA in the caudate nucleus tended to decrease with an increasing dose of DOPA, although statistically significant differences were not obtained. ^{3}H-NA in the caudate nucleus showed no changes. In the remainder of the brain also, ^{3}H-DA tended to decrease but again not significantly, since the scatter was relatively large. ^{3}H-NA, on the other hand, showed a distinct fall, particularly when the dose of DOPA was increased from 10 to 100 mg/kg. Here the yield of ^{3}H-NA further decreased by about 3 times ($P < 0.005$). A threefold reduction in the yield of ^{3}H-NA was already observed in the heart with a dose of 10 mg/kg of DOPA ($P < 0.001$). There were no changes in the ^{3}H-DA in this organ.

Effect of DOPA on the levels of NA and DA in the brain and heart.

Unlabelled DOPA, 1 or 100 mg/kg, was given intravenously to mice. Two hours later the animals were killed. The NA and DA in the caudate nucleus, the remainder of the brain and the heart were determined. Untreated animals served as controls. No significant changes in the catecholamine levels of the caudate nucleus could be observed after DOPA (table 3), although there was a tendency to a slight increase of both NA and DA. Whilst NA in the remainder of the brain did not change, DA was increased by about 30 per cent ($P < 0.001$) after 100 mg/kg of DOPA. In the heart NA showed a slight increase after a dose of 1 mg/kg DOPA ($P < 0.05$).

Table 2.

^{3}H noradrenaline (^{3}H-NA) and ^{3}H-dopamine (^{3}H-DA) in the brain and heart after ^{3}H tyrosine and different doses of unlabelled DOPA. Mice received 10 µg/kg ^{3}H-tyrosine together with unlabelled DOPA intravenously. One hour later the animals were killed. The ^{3}H-NA and ^{3}H-DA in the caudate nucleus, the remainder of the brain and the heart were determined. The mean ± S. E. M. of 6–7 experimental groups, each comprising 6 animals are shown. The data are expressed in fmoles (10^{-15} mol) per gram tissue.

Unlabelled DOPA mg/kg	Caudate nucleus		Remainder of brain		Heart	
	^{3}H-NA	^{3}H-DA	^{3}H-NA	^{3}H-DA	^{3}H-NA	^{3}H-DA
0	37 ± 5	1044 ± 200	47 ± 4	83 ± 16	61 ± 8	132 ± 42
10	41 ± 7	949 ± 157	35 ± 5	78 ± 16	21 ± 2	118 ± 41
100	36 ± 6	692 ± 170	13 ± 2	59 ± 11	19 ± 8	127 ± 40

Data for the heart seem to indicate increased levels of DA after DOPA. However, the values are near the analytical limit of the method and not statistically significant.

Discussion

When increasing amounts of unlabelled DOPA were added to one and the same tracer dose of ^{3}H-DOPA, the yield of NA and DA formed from this amino acid in the brain and heart, rose at first almost linearly, as indicated by the constant yield of ^{3}H-NA and ^{3}H-DA. With doses of DOPA between 10 and 100 mg/kg this course was no longer found for NA in the brain, the

Table 3.

Noradrenaline (NA) and dopamine (DA) in the brain and heart after different doses of DOPA. Mice received DOPA intravenously in various doses. Two hours later the animals were killed. The NA and DA in the caudate nucleus, the remainder of the brain and the heart were determined. The mean ± S. E. M. of 6 experimental groups, each comprising 6 animals are shown. The data are expressed in µg/g tissue.

DOPA mg/kg	Caudate nucleus		Remainder of brain		Heart	
	NA	DA	NA	DA	NA	DA
0	0.20 ± 0.07	7.0 ± 0.6	0.57 ± 0.04	0.40 ± 0.03	0.73 ± 0.04	0.05 ± 0.03
1	0.29 ± 0.02	7.5 ± 0.4	0.56 ± 0.03	0.45 ± 0.01	0.98 ± 0.08	0.22 ± 0.10
100	0.30 ± 0.10	7.7 ± 0.3	0.63 ± 0.03	0.54 ± 0.02	0.94 ± 0.09	0.25 ± [illegible]

relative yield of this amine being reduced. In the heart this change was already evident with doses between 1 and 10 mg/kg of DOPA. Since the formation of DA from DOPA did not appear to cease, saturation of the DA β-hydroxylase seems to be the most probable reason for the reduced yield of NA.

The penetration of DOPA across the blood-brain barrier appears to be relatively poor, partly due to decarboxylation in the capillary walls of the brain (BERTLER *et al.* 1966; CONSTANTINIDIS *et al.* 1969; BARTHOLINI & PLETSCHER 1968). This may explain why a retarded NA synthesis was observed rather in the heart than in the brain with the lower doses.

In agreement with previous investigations (BURACK & DRASKÓZY 1964; BARTHOLINI & PLETSCHER 1968; PERSSON & WALDECK 1968), the yield of DA in the brain after moderate doses of labelled DOPA was lower than that of NA, in relation to their endogenous levels. When labelled tyrosine is used a more "physiological" ratio is obtained (see also UDENFRIEND & ZALTZMAN NIRENBERG 1963). This relatively poor yield of DA after labelled L-DOPA as compared to that following L-tyrosine has also been demonstrated in the caudate nucleus (PERSSON 1969), indicating that the differences observed involved a different transmitter yield in DA and NA containing neurons.

The increase in the yield of ^{3}H-DA in the caudate nucleus with increasing doses of DOPA (table 1) may be due to saturation of a mechanism which in one way or another reduces the yield; for example the DOPA decarboxylase of the barrier (see above), catechol-O-methyl transferase, or monoamine oxidase. The difference which appears to exist between DA and NA neurons in this respect may have several possible reasons. It should be recalled that there is a difference in the amine uptake mechanisms of the cell membrane between NA and DA neurons, only the former being efficiently blocked by e.g. tricyclic antidepressives (CARLSSON *et al.* 1966; HÄGGENDAL & HAMBERGER 1967).

The formation of ^{3}H-NA both from ^{3}H-tyrosine *and from* ^{3}H-DOPA was *influenced in a similar manner by DOPA. Thus, in both cases,* the reduction in the yield of ^{3}H-NA occurred with the same dose of DOPA, and was lower in the brain than in the heart. These data suggest that after increasing doses of DOPA, the DA β-hydroxylase is the first enzyme to approach saturation.

In the brain, the yield of ^{3}H-DA from ^{3}H-tyrosine tended to decrease with high doses of DOPA, especially in the caudate nucleus. This *demonstrates* an effect of DOPA on the endogenous synthesis of *DA in* DA containing neurons. The effect may be due to an *inhibitory action* of DOPA, its metabolites, or both, *on tyrosine hydroxylase or on* the transport of tyrosine into *the neurons.*

A significant increase in the endogenous level of DA could not be observed in the caudate nucleus two hours after 100 mg/kg of DOPA. *Under*

these conditions 20–30 per cent of the total DA should have been derived from the injected DOPA as calculated from table 1. In the remainder of the brain, DOPA caused a slight but significant increase in DA. To what extent this excessive DA is located in the catecholamine neurones cannot be elucidated at present. DOPA may also be decarboxylated in 5-HT neurones. In the heart excessive amounts of DA formed after DOPA administration, may originate e. g. from mast cells.

Both in the brain and heart the NA derived from 100 mg/kg DOPA was roughly 30 per cent of the total (c. f. fig. 1 and table 1); only the heart showed an elevation of the normal level. This difference between the brain and heart may be explained by the slower turn-over of NA in the latter organ.

Acknowledgements

This investigation was supported by the Swedish Medical Research Council (B70-14X-155-06B), Göteborgs Läkaresällskap and Göteborgs och Bohus Läns Landsting. We are grateful for the expert technical assistance given by Mrs. Barbro Aldäng, Miss Laila Johansson and Mrs. Lena Löfberg.

REFERENCES

Bartholini, G. & A. Pletscher: Cerebral accumulation and metabolism of ^{14}C-DOPA after selective inhibition of peripheral decarboxylase. *J. Pharm exp. Ther.* 1968, **161**, 14–20.

Bertler, A., A. Carlsson & E. Rosengren: A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958, **44**, 273–292.

Bertler, A., B. Falck, Ch. Owman & E. Rosengren: The localisation of monoaminergic blood-brain barrier mechanisms. *Pharm. Rev.* 1966, **18**, 369–385.

Bertler, A. & E. Rosengren: On the distribution in brain of monoamines and of enzymes responsible for their formation. *Experientia* 1959, **15**, 382.

Burack, W. R. & P. R. Draskóczy: The turnover of endogenously labelled catecholamine in several regions of the sympathetic nervous system. *J. Pharm. exp. Ther.* 1964, **144**, 66–75.

Carlsson, A., K. Fuxe, B. Hamberger & M. Lindqvist: Biochemical and histochemical studies on the effects of imipramine-like drugs and (+)-amphetamine on central and peripheral catecholamine neurons. *Acta physiol. scand.* 1966, **67**, 481–497.

Carlsson, A. & M. Lindqvist: *In vivo* decarboxylation of α-methyl DOPA and α-methyl tyrosine. *Acta physiol. scand.* 1962, **54**, 87–94.

Carlsson, A. & B. Waldeck: A fluorimetric method for the determination of dopamine (3-hydroxytyramine). *Acta physiol. scand.* 1958, **44**, 293–298.

Constantinidis, J., J. C. de la Torre, R. Tissot & F. Geissbuhler: La barrière capillaire pour la dopa dans le cerveau et les différents organes. *Psychopharmacologia* (Berlin) 1969, **15**, 75–87.

Häggendal, J. & B. Hamberger: Quantitative *in vitro* studies on noradrenaline and its inhibition by amphetamine, desipramine and chlorpromazine. *Acta p[illegible] scand.* 1967, **70**, [illegible]

Persson, T.: Central and peripheral catecholamine turnover studied by means of ^{3}H-DOPA and ^{3}H-tyrosine. *Acta pharmacol. et toxicol.* 1969, **27**, 397–409.

Persson, T. & B. Waldeck: The use of ^{3}H-DOPA for studying cerebral catecholamine metabolism. *Acta pharmacol. et toxicol.* 1968, **26**, 363–372.

Persson, T. & B. Waldeck: A source of error in catecholamine turnover studies with labelled DOPA: Slow disappearance of the precursor. *Acta pharmacol. et toxicol.* 1970, **28**, 466–476.

Svensson, T. & B. Waldeck: On the significance of central noradrenaline for motor activity: Experiments with a new dopamine β-hydroxylase inhibitor. *Eur. J. Pharmacol.* 1969, **7**, 278–282.

Udenfriend, S. & P. Zaltzman-Nirenberg: Norepinephrine and 3,4-dihydroxyphenylethylamine turnover in guinea pig brain *in vivo*. *Science* 1963, **142**, 394–396.

Acta pharmacol. et toxicol. 1971, 29, 533–541.

From the Institute of Pharmacology, University of Oslo, and Institute of Aviation Medicine, Royal Norwegian Air Force, Oslo, Norway

Effects of Chlorpromazine and Propranolol on Left Ventricular Systolic Pressure, ECG, and K^+ Efflux in the Isolated Perfused Rat Heart

By

Asbjorn Langslet and Morten Ryg

(Received October 20, 1970)

Abstract: Isolated rat hearts perfused with a perfusate from which K^+ had been omitted and replaced by Rb^+ had a spontaneous rate of contraction which was about 5 % slower than that of the hearts perfused with K^+-containing perfusate, while the electrically driven hearts had a contractile force which was about 10 % lower. Isoprenaline and glucagon caused a maximal increase in contractile force of 60 % and 50 % respectively which is equal to the increase obtained with a K^+-containing perfusate. Chlorpromazine (C), d,l-propranolol (d,l-P) and d-propranolol (d-P) in concentrations from 10^{-5} M to 5×10^{-5} M caused dose dependent decreases in a) the heart rate from 135 ± 7 beats/min. (mean ± S D) (4 controls) to a minimum of 62 (C), 61 (d,l-P) and 60 (d-P). b) impulse conduction velocity expressed as PQ^{-1} from 12 ± 2.1 sec. (4 controls) to a minimum of 6 6 (C), 4 9 (d,l-P) and 4.8 (d-P). c) left ventricular systolic pressure from 95 ± 3 mmHg (4 controls) to a minimum of 56 (C), 45 (d,l-P) and 44 (d-P) d) potassium efflux from 4.2 ± 0.2 meq./g dry weight $\times 10^{-2}$ (8 controls) to a minimum of 2.9 (C), 2.7 (d,l-P) and 2 6 (d-P) It is suggested that the effects can be explained as secondary to drug induced changes in the cell membrane.

Key-words: Perfused rat heart – chlorpromazine – propranolol – membrane stabilizers.

Several investigators have described a positive correlation between the effects on the cardiac electrical and mechanical properties and the effects on ion flux induced by a variety of membrane stabilizing drugs, e. g. neuroleptics, thymoleptics, anaesthetics, barbiturates, quinidine, and β-adrenoceptor blockers (HOLLAND 1957; SHANES 1958a and b; HOLLAND *et al.* 1959; KLEIN *et al.* 1960; NAYLER 1967, NAYLER *et al.* 1969; VAN ZWIETEN 1969; LANGSLET 1970a and b).

In previous experiments we have studied drug induced changes on K^+-efflux from hearts perfused with a K^+ – and Ca^{++}-free perfusate (LANGSLET 1970a and b). In these experiments K^+ was omitted in order the K^+-gradient between the intracellular and extracellular

to prevent the active uptake of this ion during the perfusion. It was als necessary to remove Ca^{++} in order to maintain sufficient coronary flow in th absence of K^{+} (LANGSLET 1970a). This perfusate is highly "unphysiological and when this is perfused, the hearts stop beating and cannot even be drive electrically.

Recently VAN ZWIETEN (1968) demonstrated that isolated atria suspende in K^{+}-free, Rb^{+}-containing Tyrode solution showed normal mechanical ac tivity. We therefore decided to study the effects of the membrane stabilizer d,l-propranolol, d-propranolol (proprasylytum NFN) and chlorpromazine o the ECG, the cardiac contractile force and K^{+} efflux in isolated heart perfused with a buffer containing Ca^{++} and in which K^{+} was replaced by Rb^{+}

Material and Methods

Female Wistar albino rats weighing about 200 g were used as heart donors and the hearts were excised under ether anaesthesia. The methods used for heart perfusion are described in detail elsewhere (LANGSLET 1969; LANGSLET 1970b).

During the initial perfusion period (10 min.) the hearts were perfused with a modified Krebs Ringer bicarbonate solution (LANGSLET 1970a) which was not recirculated. Following this, the hearts were transferred to the recirculation unit. The recirculated perfusate (40 ml) contained 180 mg/100 ml of glucose and the following ions (meq./l): Rubidium: 5.3, sodium 143.4, calcium 5.1, magnesium 2.3, chloride 126 4, phosphate H_2PO_4 2.4, bicarbonate 25, and sulphate 2.3. Both perfusates were continuously aerated with 95 % O_2 and 5 % CO_2.

A. Perfusion of the isolated spontaneously beating heart at 32°.

The hearts were perfused in the recirculating circuit for 10 min. before the test compounds were applied. After this either saline, d,l-propranolol, d-propranolol or chlorpromazine was added, and the perfusion carried on for another 10 min. The coronary flow was measured and the ECG-tracings were recorded at regular intervals.

B. Perfusion of the isolated electrically driven heart at 32°.

The hearts were electrically driven with square wave pulses (12 V, 0.25 msec.) at 180 beats/min. Ten min. after initiation of the perfusion in the recirculating circuit either water, d,l-propranolol, d-propranolol or chlorpromazine was added, and the perfusion carried on for another 10 min. The left ventricular pressure was continuously recorded and the coronary flow measured at regular intervals.

In order to test the preparation either isoprenaline 2×10^{-9} M (2 hearts) or glucagon 2×10^{-6} M (2 hearts) was added.

C. Perfusion of the isolated asystolic heart at 16°.

Preliminary studies with electrically driven hearts at 32° showed that all the drug caused a dose dependent decrease in potassium loss. Since this loss paralleled th decrease in the left systolic pressure, the potassium loss could be due to a reduction i a possible squeezing effect of the contractions. The potassium loss was therefore studie in hearts kept in an asystolic state by lowering the temperature to 16°.

The hearts were perfused for 20 min. with and without the added drugs. The coronar flow was measured and samples for K^{+} analyses were removed at regular intervals. Th

total K⁺ loss from the hearts during the perfusion period was calculated in meq./g.d.w. (g.d.w. = gram dry weight: the hearts dried at 95° for 48 hours).

D *Instruments, assays and drugs.*

A Biotron® square wave laboratory stimulator was used for artifical pacing of the hearts. A Sanborn® transducer connected to a Hewlett Packard 7702B recorder was used for measuring the left ventricular systolic pressure. The Hewlett Packard recorder was used for ECG registrations.

K⁺ was measured by a Zeiss flame spectrophotometer for atomic absorption.

d,l-Propranolol and d-propranolol were obtained from I. C. I. (through Scanmeda) and chlorpromazine from Dumex. The drugs were dissolved in saline.

Results

A. Drug effects on the spontaneously beating and the electrically driven heart.

The non-stimulated hearts showed a sinus rhythm with constant rate during the first 1–2 min. of perfusion. The electrically stimulated hearts followed the artificial pacemaker and showed a constant left ventricular contractile force. About 4 min. after switching to the Rb⁺-perfusate the rate decreased by about 5 % (spontaneously beating hearts) and the left ventricular contractile force by about 10 % (electrically driven hearts) in 2–3 min., but then remained constant. The controls maintained a constant rate without any change in the ECG and a constant left ventricular contractile force

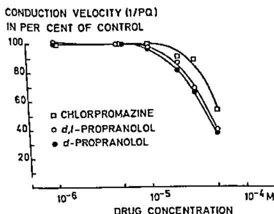


Fig. 1. Dose response curves (based on ECG-tracings) for drug induced decrease in conduction velocity (expresses as $1/PQ = PQ^{-1}$). Each point represents one experiment and shows the 1/PQ 5 min. after addition of the drug calculated in 1/PQ before the addition of the drug. 6 hearts received chl tol, and 6 d-propranolol. Saline was added to 4 hearts figure). No change in the 1/PQ was observed in these

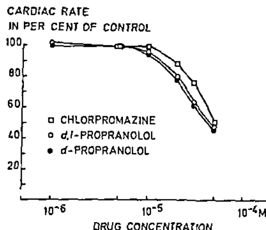


Fig. 2. Dose response curves for drug induced decreases in cardiac rate. Each point represents one experiment and shows the rate 5 min. after the addition of the drug calculated in per cent of the rate before addition of the drug. The curves are based on ECG-tracings in the same experiments as described in the legend to fig. 1. No change in the rate was observed in the 4 control hearts.

during the remaining perfusion period. All the drugs at concentrations above 10^{-5} M caused a dose dependent increase in the PQ-interval (fig. 1) and a decrease in rate (fig. 2). At concentrations above 5×10^{-5} M partial and

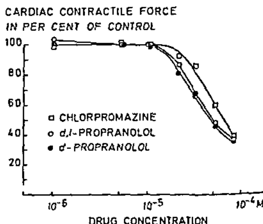


Fig. 3. Dose response curves (based on left ventricular pressure recordings) for drug induced decrease in cardiac contractile force. Each point represents one experiment and shows the contractile force 5 min. after addition of the drug calculated in per cent of the contractile force before addition of the drug. 7 hearts received *chlorpromazine*, 7 d,l-propranolol, and 7 d-propranolol. *Saline was added to 4 hearts (controls, not shown in the figure).* No change in contractile force was observed in these control hearts.

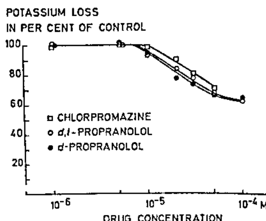


Fig. 4. Dose response curves for drug induced decrease in K^+ loss from asystolic hearts. Each point represents one experiment and shows the total K^+ loss (perfusion time [illegible] min.) calculated in per cent of the total loss (mean) from 7 control hearts (no drug added). Six hearts received chlorpromazine, 7 d,l-propranolol, and 7 d-propranolol. Only minor change in the coronary flow (increase) were induced by the drugs in the concentration range shown in the figure (at concentrations above 5×10^{-5} M, chlorpromazine decreased the flow under the present experimental conditions).

later total AV-block appeared. All the drugs caused a dose dependent decrease in left ventricular systolic pressure (contractile force) (fig. 3).

At concentrations above 10^{-5} M d- and d,l-propranolol an increase of coronary flow was found. In addition chlorpromazine decreased the flow in concentrations above 5×10^{-5} M.

Isoprenaline (2×10^{-8} M) and glucagon (2×10^{-6} M) respectively caused about 60 and 50 per cent increase in cardiac contractile force.

B. Drug effects on K^+ efflux from the asystolic heart.

The hearts of this series were perfused at 16° and did not contract during the perfusion period. The ECG too showed no electrical activity. In previous experiments with Ca^{++}- and K^+-free buffer, the coronary flow did not change on addition of the drugs (Langslet 1970a and b). When perfusion was performed with this Rb^+-, Ca^{++}-containing perfusate, a fall in the coronary flow appeared at concentrations of chlorpromazine above 5×10^{-5} M. Propranolol (d,- and d,l) did not decrease the flow in any of the concentrations tested. At lower concentrations all the drugs slightly increased the flow.

As shown in fig. 4 the drugs tested reduced the amount of K^+ loss from the hearts. d,l-Propranolol, d-propranolol and chlo[illegible] in this respect.

interface. NAYLER *et al.* (1969) recently showed that several β-adrenoceptor blockers including propranolol impede this transport and that this effect parallels their negative inotropic effect.

The molecular mechanisms of the drug action however, are poorly understood. It has been suggested that the stabilizers change either the proteins (MIZUSHIMA & KOBAYASHI 1968; MIZUSHIMA & SAKAI 1969; MIZUSHIMA *et al.* 1970), the lipids (SHANES 1963; SUAREZ-KURTZ *et al.* 1970), the lipid-protein interface (HUBELL *et al.* 1969) or displace ions from binding sites in the membrane (KWANT & SEEMAN 1969; SUAREZ-KURTZ *et al.* 1970). The drugs thus alter the physical properties of the cell membrane by one or more of these changes. This alteration in the physical properties of the membrane may make the cell less excitable and the membrane less permeable, the latter phenomenon being parallel to the former and not the cause of it. The fact that membrane stabilizers decrease permeability to macromolecules in the liver (FONKALSRUD *et al.* 1969) and heart (unpublished observations in our laboratories) lends support to this hypothesis.

Acknowledgement

We would like to thank Dr. I. Øye of the Institute of Pharmacology and Dr. F. Vogt Lorentzen of the Institute of Aviation Medicine for their help. We are grateful to Miss Lilly Skutle for her technical assistance. Finally we wish to thank Scanmeda (I. C. I.) and Dumex for their supply of drugs.

REFERENCES

Feinstein, M. B.: Reaction of local anesthetics with phospholipids. *J. Gen. Physiol.* 1964, 48, 357–374.

Fonkalsrud, E. W., D. M Rangel, J. Byfield, W. Bruckner, G. H. Stevens & A. Dinbar: Hepatic preservation with chlorpromazine and phenoxybenzamine; application to liver transplantation *Surgery* 1969, 66, 23–33.

Holland, W. C.: A possible mechanism of action of quinidine. *Amer J. Physiol.* 1957, 190, 492–494.

Holland, W C, R L. Klein & A. H. Briggs: Factors affecting the action of acetyl choline on transmembrane flux of K in isolated rabbit atria *Amer. J. Physiol* 1959, 196, 478–482.

Hubell, W. L., H M McConell & J. C. Metcalfe· Magnetic resonance studies of anaesthetics in cyto-membranes. *Brit. J Pharmacol* 1969, 35, 374–375.

Klein, R. L., W. C Holland & B. Tinsley: Quinidine and cation fluxes in atria. *Circ Res* 1960, 8, 246–253.

Kwant, W. O. & P. Seeman: The displacement of membrane calcium by a local anesthetic (chlorpromazine). *Biochim Biophys. Acta* 1969, 193, 338–349.

Langslet, A.: Changes in coronary flow and ECG in the isolated perfused rat heart induced by phenothiazine drugs. *Acta pharmacol. et toxicol.* 1969, 27, 183–192.

Langslet, A.: Cardiac effect of antiparkinson drugs, local anesthetics, and tranquilizers. *Eur. J. Pharmacol.* 1970a, 9, 269–275.

Langslet, A.: Membrane stabilization and cardiac effects of d,l-propranolol, d-propranolol and chlorpromazine. *Eur. J. Pharmacol.* 1970b, 9, in press.
Mizushima, Y. & M. Kobayashi: Interaction of antiinflammatory drugs with serum proteins, especially with some biologically active proteins. *J. Pharm. Pharmacol.* 1968, 20, 169–173.
Mizushima, Y. & S. Sakai: Stabilization of erythrocyte membrane by non-steroid anti-inflammatory drugs. *J. Pharm. Pharmacol.* 1969, 21, 327–329.
Mizushima, Y., S. Sakai & M. Yamaura: Mode of stabilizing action of non-steroid anti-inflammatory drugs on erythrocyte membrane. *Biochem. Pharmacol.* 1970, 19, 227–234.
Nayler, Winifred G.: Calcium exchange in cardiac muscle: A basic mechanism of drug action. *Amer. Heart J.* 1967, 73, 379–394.
Nayler, Winifred G., J. Stone, Valerie Carson, I. McInnes, Valerie Mack & T. E. Lowe: The effect of beta-adrenergic antagonists on cardiac contractions, myofibrillar ATPase activity, high-energy phosphate stores and lipid-facilitated transport of calcium ions. *J Pharmacol. Exp. Therap* 1969, 165, 225–233.
Shanes, A. M.: Electrochemical aspects of physiological and pharmacological action in excitable cells. Part I. *Pharmacol. Rev.* 1958a, 10, no. 1, 59–164.
Shanes, A. M.: Electrochemical aspects of physiological action in excitable cells. Part II. *Pharmacol. Rev.* 1958b, 10, no. 2, 166–273.
Shanes, A. M.: Drugs and nerve conduction. *Ann. Rev. Pharmacol.* 1963, 3, 185–204.
Suarez-Kurtz, G., C. P. Bianchi & P. Krupp: Effect of local anesthetics on radiocalcium binding in nerve. *Eur. J. Pharmacol.* 1970, 10, 91–100.
Van Zwieten, P. A.: The use of ^{86}rubidium for the determination of changes in membrane permeability in guinea pig atrial tissue. *Pflügers Arch. Ges. Physiol.* 1968, 303, 81–98.
Van Zwieten, P. A.: Decrease in ionic permeability of the cell membrane in guinea-pig atrial tissue by treatment with antifibrillatory agents and hexobarbitone, determined by means of ^{86}Rb. *Brit. J. Pharmacol.* 1969, 35, 103–111.

From the Departments of Histology, Pharmacology, and Psychiatry II,
University of Lund, Sweden

Effect of Chlorpromazine, Desmethylimipramine and Lithium on Dopamine Uptake in the Rat Pancreas

By

Lars Nordgren

(Received December 10, 1970)

Key-words: Catecholamines – chlorpromazine – imipramine.

The uptake of catecholamines by the adrenergic neuron is inhibited by some psychotropic drugs (Axelrod *et al.* 1962) but very little is known about their possible interference with catecholamine uptake in non-neuronal tissues. In this paper some observations are described on the effect of chlorpromazine (CPZ), desmethylimipramine (DMI) and lithium on the uptake of dopamine (DA) in the pancreatic cells. The exocrine pancreas was chosen as a model system because it possesses an efficient uptake mechanism for dopa; DA is also taken up, though not so readily (Alm *et al.* 1969).

CPZ-HCl (25 mg/kg) or DMI-HCl (25 mg/kg) was given intraperitoneally to adult rats 2 hrs before the intravenous administration of DA-HCl (40 mg/kg). Lithium carbonate (8 meq./kg) was given by stomach tube 10 and 2 hrs before the injection of DA. After sacrifice blood samples for flame photometrical determination of lithium were collected from each animal. Rats receiving only DA served as controls. All the animals were kept at 32° from about 2 hrs before being killed 10 min. after the DA injections. A small piece was dissected out from each pancreatic gland and processed for fluorescence microscopy according to the method of Falck & Hillarp (see Falck & Owman 1965). The pancreatic concentration of DA in each animal was determined according to Bertler *et al.* (1958).

Ten minutes after the injection of DA, considerable amounts of the amine were found in the pancreas. However, after pretreatment with CPZ or DMI, this amount was significantly reduced, the two drugs being equally potent in this respect. Pretreatment with lithium produced no effect.

In the animals receiving only DA, the exocrine and the endocrine cells as expected (Alm *et al.* 1969), displayed in the fluorescence microscope, a

Table 1.

Influence of chlorpromazine (CPZ), desmethylimipramine (DMI) and lithium treatment on dopamine (DA) uptake in rat pancreas. Figures in brackets indicate number of rats.

Treatment	DA
DA 40 mg/kg i. v. 10 min.	μg/g wet weight ± S.D. 11.0 ± 2.3 (6)
CPZ 25 mg/kg i. p. 2 hrs. before DA	2.5* ± 1.2 (5)
DMI 25 mg/kg i. p. 2 hrs. before DA	2.8* ± 0.96 (4)
Lithium 8 meq./kg p. o. 10 and 2 hrs before DA**	11.1 ± 3.6 (4)

* Levels of significance compared with DA alone; ($P < 0.001$ Student's t-test).
** Lithium in blood serum; 4 3 ± 0.4 (4).

weak to moderate, diffuse, cytoplasmic catecholamine fluorescence, and a few exocrine cells contained some apical granules exhibiting a marked specific fluorescence. A moderate green fluorescence was also seen in connective tissue strands; in some specimens, the mast cells (identified by staining in toluidine blue) emitted a green-yellow light. Identical observations were made in the animals treated with lithium. After the administration of CPZ or DMI no fluorescence was seen in the exocrine and the endocrine cells, whereas the catecholamine fluorescence of the connective tissue appeared as in the animals given only DA. No mast cells were found in these tissue sections.

The chemical and histochemical analyses strongly indicate that CPZ and DMI, but not lithium can block the uptake of DA into the pancreatic cells as well as block the uptake of catecholamines in the adrenergic nerves.

It is well known that shortly after the administration of high doses of DA a transient unspecific accumulation occurs in inter alia the connective tissue. It is therefore no surprise that the green fluorescence in the pancreatic connective tissue strands appeared to be similar in all the experimental groups. This finding could also explain, at least to some extent, the pancreatic DA in animals pretreated with CPZ or DMI. It cannot be excluded that part of this DA is located in the pancreatic parenchymal cells, but diffusely distributed in concentrations too low to be detected by the fluorescence method.

DMI has been shown to be a more potent inhibitor of neuronal catecholamine uptake than CPZ (CARLSSON & WALDECK 1965; DENGLER 1965). In the present study no difference in the uptake blocking efficiency could be demonstrated. Whether or not this reflects a difference in the uptake mechanism of adrenergic nerves and pancreatic cells must analyses.

REFERENCES

Alm, P., B. Ehinger & B. Falck: Histochemical studies on the metabolism of L-Dopa and some related substances in the exocrine pancreas. *Acta physiol. Scand.* 1969, 76, 106–120.

Axelrod, J., G. Hertting & L. Potter: Effect of drugs on the uptake and release of ^{3}H-norepinephrine in the rat heart. *Nature* 1962, 4825, 297.

Bertler, A., A. Carlsson, E. Rosengren & B. Waldeck: A method for the fluorimetric determination of adrenaline, noradrenaline and dopamine in tissues. *Kungl. Fysiogr. Sällsk. Förh.* 1958, 28, 121–123.

Carlsson, A. & B. Waldeck: Inhibition of ^{3}H-metaraminol uptake by antidepressive and related agents. *J. pharm. pharmacol.* 1965, **17**, 243–244.

Dengler, H. J.: Effect of drugs on monoamine uptake in isolated tissues. In: *Pharmacology of cholinergic and adrenergic transmission.* Ed.: G. B. Koelle, W. W. Douglas and A. Carlsson. Proc. 2nd Int. Pharmacol. Meeting, Prague 1963, Pergamon Press, Oxford, 1965, 3, 261–275.

Falck, B. & C. Owman. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund* 1965, 7, 5–23.

From the Departments of Histology, Pharmacology, and Psychiatry II,
University of Lund, Sweden

Effect of Chlorpromazine, Desmethylimipramine and Lithium on Dopamine Uptake in the Rat Pancreas

By

Lars Nordgren

(Received December 10, 1970)

Key-words: Catecholamines – chlorpromazine – imipramine.

The uptake of catecholamines by the adrenergic neuron is inhibited by some psychotropic drugs (AXELROD *et al.* 1962) but very little is known about their possible interference with catecholamine uptake in non-neuronal tissues. In this paper some observations are described on the effect of chlorpromazine (CPZ), desmethylimipramine (DMI) and lithium on the uptake of dopamine (DA) in the pancreatic cells. The exocrine pancreas was chosen as a model system because it possesses an efficient uptake mechanism for dopa; DA is also taken up, though not so readily (ALM *et al.* 1969).

CPZ-HCl (25 mg/kg) or DMI-HCl (25 mg/kg) was given intraperitoneally to adult rats 2 hrs before the intravenous administration of DA-HCl (40 mg/kg). Lithium carbonate (8 meq./kg) was given by stomach tube 10 and 2 hrs before the injection of DA. After sacrifice blood samples for flame photometrical determination of lithium were collected from each animal. Rats receiving only DA served as controls. All the animals were kept at 32° from about 2 hrs before being killed 10 min. after the DA injections. A small piece was dissected out from each pancreatic gland and processed for fluorescence microscopy according to the method of FALCK & HILLARP (see FALCK & OWMAN 1965). The pancreatic concentration of DA in each animal was determined according to BERTLER *et al.* (1958).

Ten minutes after the injection of DA, considerable amounts of the amine were found in the pancreas. However, after pretreatment with CPZ or DMI, this amount was significantly reduced, the two drugs being equally potent in this respect. Pretreatment with lithium produced no effect.

In the animals receiving only DA, the exocrine and the endocrine cells as expected (ALM *et al.* 1969), displayed in the fluorescence microscope, a

Table 1.

Influence of chlorpromazine (CPZ), desmethylimipramine (DMI) and lithium treatment on dopamine (DA) uptake in rat pancreas. Figures in brackets indicate number of rats.

Treatment	DA
DA 40 mg/kg i. v. 10 min.	μg/g wet weight ± S.D. 11.0 ± 2.3 (6)
CPZ 25 mg/kg i. p. 2 hrs. before DA	2 5* ± 1 2 (5)
DMI 25 mg/kg i. p. 2 hrs. before DA	2.8* ± 0.96 (4)
Lithium 8 meq./kg p. o. 10 and 2 hrs before DA**	11 1 ± 3 6 (4)

* *Levels of significance compared with DA alone; (P < 0 001 Student's t-test).*
** Lithium in blood serum; 4.3 ± 0.4 (4).

weak to moderate, diffuse, cytoplasmic catecholamine fluorescence, and a few exocrine cells contained some apical granules exhibiting a marked specific fluorescence. A moderate green fluorescence was also seen in connective tissue strands; in some specimens, the mast cells (identified by staining in toluidine blue) emitted a green-yellow light. Identical observations were made in the animals treated with lithium. After the administration of CPZ or DMI no fluorescence was seen in the exocrine and the endocrine cells, whereas the catecholamine fluorescence of the connective tissue appeared as in the animals given only DA. No mast cells were found in these tissue sections.

The chemical and histochemical analyses strongly indicate that CPZ and DMI, but not lithium can block the uptake of DA into the pancreatic cells as well as block the uptake of catecholamines in the adrenergic nerves.

It is well known that shortly after the administration of high doses of DA a transient unspecific accumulation occurs in inter alia the connective tissue. It is therefore no surprise that the green fluorescence in the pancreatic connective tissue strands appeared to be similar in all the experimental groups. This finding could also explain, at least to some extent, the pancreatic DA in animals pretreated with CPZ or DMI. It cannot be excluded that part of this DA is located in the pancreatic parenchymal cells, but diffusely distributed in concentrations too low to be detected by the fluorescence method.

DMI has been shown to be a more potent inhibitor of neuronal catecholamine uptake than CPZ (Carlsson & Waldeck 1965; Dengler 1965). In the present study no difference in the uptake blocking efficiency could be demonstrated. Whether or not this reflects a difference in the uptake mechanism of adrenergic nerves and pancreatic cells must await further analyses.

REFERENCES

Alm, P., B. Ehinger & B. Falck: Histochemical studies on the metabolism of L-Dopa and some related substances in the exocrine pancreas. *Acta physiol. Scand.* 1969, 76, 106–120.

Axelrod, J., G. Hertting & L. Potter: Effect of drugs on the uptake and release of ^{3}H-norepinephrine in the rat heart. *Nature* 1962, 4825, 297.

Bertler, Å., A. Carlsson, E. Rosengren & B. Waldeck: A method for the fluorimetric determination of adrenaline, noradrenaline and dopamine in tissues. *Kungl. Fysiogr. Sällsk. Förh.* 1958, 28, 121–123.

Carlsson, A. & B. Waldeck: Inhibition of ^{3}H-metaraminol uptake by antidepressive and related agents. *J. pharm. pharmacol.* 1965, 17, 243–244.

Dengler, H. J.: Effect of drugs on monoamine uptake in isolated tissues. In: *Pharmacology of cholinergic and adrenergic transmission.* Ed.: G. B. Koelle, W. W. Douglas and A. Carlsson. Proc. 2nd Int. Pharmacol. Meeting, Prague 1963, Pergamon Press, Oxford, 1965, 3, 261–275.

Falck, B. & C. Owman: A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund* 1965, 7, 5–23.